

1 **Community sequencing on a natural experiment reveals little influence of host species and**  
2 **timing but a strong influence of compartment on the composition of root endophytes in three**  
3 **annual Brassicaceae**

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14

15 **Abstract**

16 The plant family Brassicaceae includes some of the most studied hosts of plant microbiomes,  
17 targeting microbial diversity, community assembly rules, and effects on host performance.

18 Compared to bacteria, eukaryotes in the brassicaceous microbiome remain understudied, especially  
19 under natural settings. Here, we assessed the impact of host identity and age on the assembly of  
20 fungal and oomycete root communities, using DNA metabarcoding of roots and associated soil of  
21 three annual co-habiting Brassicaceae collected at two time points. Our results showed that fungal  
22 communities are more diverse and structured than those of oomycetes. In both cases, plant identity  
23 and sampling time had little influence on community variation, whereas root/soil compartment had  
24 a strong effect by exerting control on the entry of soil microorganisms into the roots. The  
25 enrichment in roots of specific fungi suggests a specialization towards the asymptomatic  
26 colonization of plant tissues, which could be relevant to host's fitness and health.

27  
28 **Keywords:** Brassicaceae, endophytes, microbiome, pathogens, roots, symbiosis

29

## 30 **Introduction**

31 Plants associate through their roots with microbial communities that are essential for their fitness,  
32 health, and response to environmental cues (Carrión et al., 2019; Durán et al., 2018; Kia et al.,  
33 2017). The elucidation of the mechanisms responsible for these associations, as well as the factors  
34 affecting them, provide new perspectives about how plant communities and ecosystems function  
35 (Klein et al., 2016; Wagg et al., 2019), and will likely enable the exploitation in agriculture of  
36 multiple aspects of plant-microbe symbioses (Duhamel and Vandenkoornhuyse, 2013; Wei and  
37 Jousset, 2017). Research focused on several members of the plant family Brassicaceae has  
38 spearheaded the advances in knowledge about the plant-associated microbiota (a.k.a. microbiomes)  
39 diversity and function (e.g., Almario et al., 2017; Bulgarelli et al., 2012; Dombrowski et al., 2016;  
40 Durán et al., 2018; Glynou et al., 2018a, 2018b; Keim et al., 2014; Lundberg et al., 2012; Wagner et  
41 al., 2016), both because of their economic importance and their inclusion of the most studied plant  
42 model species, *Arabidopsis thaliana* (L.) Heynh.

43 The microbiome of Brassicaceae species comprises multi-kingdom microbial communities  
44 dominated by bacteria, fungi, and oomycetes, which closely interact both with the host and among  
45 each other (Hassani et al., 2018). Most studies have focused on bacteria, as they may represent the  
46 largest fraction of the plant microbiome, have important effects on host performance, and have been  
47 recently proven instrumental in keeping at bay the detrimental effects caused by endophytic fungi  
48 and oomycetes on plant growth (Bulgarelli et al., 2012; Dombrowski et al., 2016; Durán et al.,  
49 2018; Lundberg et al., 2012; Wagner et al., 2016). However, the latter filamentous eukaryotes can  
50 also play important roles in microbiome function, encompassing likely keystone taxa that determine  
51 the structure of the microbial communities (Agler et al., 2016), well known pathogens that impact  
52 plant health and productivity, and mutualists able to assist hosts in the acquisition of nutrients  
53 (Almario et al., 2017; Hiruma et al., 2016).

54 Multiple studies have helped reveal the diversity and factors affecting the assembly of the  
55 eukaryotic microbiome of brassicaceous hosts, especially of fungi. However, a better resolution of

56 the predominant fungi and oomycetes occurring in plants under natural conditions is still needed.  
57 Multiple studies engage in single sampling, have low spatial resolution with respect to the plant  
58 compartment investigated, or focus on common gardens with conditions different to those in natural  
59 habitats (but, for exceptions, see Glynou et al., 2018a, 2016; Thiergart et al., 2019). Besides,  
60 important factors that may affect the assembly of plant-associated microbial communities, such as  
61 plant identity and age or phenological status, have been addressed in bacteria (Dombrowski et al.,  
62 2016; Schlaeppi et al., 2014) but remain understudied in fungi and oomycetes. For example, the  
63 effect of brassicaceous host plant species in microbiome assembly has been mainly investigated  
64 across genotypes within a species (Agler et al., 2016; Glynou et al., 2016; Urbina et al., 2018), or  
65 using limited experimental settings that preclude generalization (Glynou et al., 2018b). The effect of  
66 time on the fungal and oomycete fraction of the microbiome has been studied across consecutive  
67 years (Thiergart et al., 2019), but finer resolution studies to evaluate the impact of host phenology  
68 are lacking.

69 Here, we address the impact of plant identity and age on the assembly of fungal and oomycete  
70 communities in root-associated soil and roots of annual Brassicaceae. We approach these questions  
71 by sampling at two time points – representing phenological growth stages of basal rosette and  
72 flowering – specimens of the brassicaceous plant species *A. thaliana*, *Cardamine hirsuta* L., and  
73 *Draba verna* L., co-habiting in a habitat undisturbed for decades. We targeted microbial  
74 communities at different rhizocompartments, ranging from bulk soil to the root endosphere, which  
75 have repeatedly shown to represent different degrees of host influence on microbial communities  
76 (so-called host-filtering effect) that increases toward the plant inner tissues (Almario et al., 2017;  
77 Bulgarelli et al., 2012; Lundberg et al., 2012; Martínez-Diz et al., 2019; Thiergart et al., 2019). We  
78 hypothesized that the effects of both host plant genotype and age on fungal and oomycete  
79 communities increases from soil to root compartments, owing to a host filtering effect on  
80 community composition that selects for specialized microorganisms with root-colonizing traits.  
81 Based on previous results on disturbed habitats, we expect a low influence of plant species in the

82 assembly of fungal and oomycete communities (Glynou et al., 2018b), and a stronger effect of plant  
83 age due to normal seasonal changes in the soil pool of microbial species, coupled with metabolic  
84 changes in the host driven by phenology.

85

## 86 **Materials and Methods**

### 87 *2.1 Sample collection and process*

88 The sampling site is located in west Germany (N 50.09844, E 8.54721, 114 m a.s.l.), consisting of  
89 an ancient gravel path in the north of the old castle of Frankfurt Hoechst, the sides of which were  
90 covered in moss that provided the habitat for various small annual plants. Populations of the  
91 Brassicaceae species *A. thaliana*, *C. hirsuta*, and *D. verna* co-habited at the site as interspersed  
92 stands, covering an area of approx. 50 m<sup>2</sup>. Samplings were performed at two time points: on  
93 February 20<sup>th</sup>, 2014, when plants were in a stage of basal rosette; and on April 20<sup>th</sup>, 2014, when  
94 plants presented a flowering stalk carrying fully developed flowers and siliques. At every sampling  
95 time, ten specimens of *A. thaliana*, and three specimens of each *C. hirsuta* and *D. verna* were  
96 collected by carefully uprooting the plants, taking care not to detach the soil particles adhered to  
97 roots. In addition, for each species, soil samples of approx. 10 cm<sup>3</sup> were collected from just beneath  
98 each plant, and from approx. 20 cm apart in a spot free of flowering plants, using a small shovel that  
99 was rinsed and disinfected with isopropanol after each sample was taken. As the bulk soil was not  
100 expected to be very dissimilar across the site, only three bulk soil samples were taken for  
101 comparison with *A. thaliana* roots. Plant and soil samples were individually stored in zip-lock bags  
102 and brought to the laboratory in the same day, where they were kept at 4 C until processing on the  
103 next day.

104 For each plant individually, roots were separated from stems and soil adhering to the roots was  
105 removed by putting the roots into 50 ml reaction tubes half-filled with a sterile 0.01 % (v/v)  
106 Tween 20 solution and shaking for 10 min. After removal of the roots, the root-associated soil  
107 (rhizosphere soil) was pelleted by centrifugation and the supernatant removed before further

108 processing. The washed roots were then separated into two halves, of which one was surface  
109 sterilized by incubating in 4 % (w/v) sodium hypochlorite solution with gentle shaking for about  
110 20 s. Soil samples were homogenized and approximately 0.5 g of soil were mixed with the lysis  
111 buffer of the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, USA) and disrupted in a mixer  
112 mill (Retsch MM 200, Retsch GmbH, Haan, Germany) for 5 min at 25 Hz, using three iron beads  
113 with 3.5 mm per 2 ml reaction vial. After processing, samples represented five different root/soil  
114 compartments, each comprising 32 samples [(10 *A. thaliana* + 3 *C. hirsuta* + 3 *D. verna*) ×  
115 sampling times]: bulk soil (soil collected apart from plant specimens), root zone soil (soil collected  
116 underneath plant specimens), rhizosphere (soil washed out of roots), root (non-sterilized roots), and  
117 endosphere (surface-sterilized roots).

118

#### 119 *DNA extraction, amplification and sequencing*

120 Total genomic DNA was extracted from root samples using the BioSprint 96 DNA Plant Kit  
121 (Qiagen GmbH, Hilden, Germany) on a KingFisher Flex 96 robotic system (Thermo Fisher  
122 Scientific, Waltham, MA, USA), and from soil samples with the FastDNA™ SPIN Kit for Soil,  
123 following the manufacturers' instructions. DNA extracts were used directly, or after 10<sup>-1</sup> dilution in  
124 molecular biology grade ddH<sub>2</sub>O (VWR Chemicals, Darmstadt, Germany) for specific amplification  
125 and high throughput sequencing of the fungal rDNA internal transcribed spacer 1 (ITS1), and the  
126 oomycete cytochrome c oxidase subunit 2 (*cox2*) gene.

127 Amplification of the fungal ITS1 was done using primers ITS1F and ITS2, modified as in Smith  
128 and Peay (2014) to include the Illumina Nextera adapters, a linker sequence, and 12-bp error-  
129 correcting Golay barcodes (Table S4). PCR reactions were done in duplicate, in volumes of 25 µl,  
130 containing 1 µl of DNA template, 1× Phusion HF buffer (New England Biolabs GmbH,  
131 Schwalbach, Germany) with 1.5 mM MgCl<sub>2</sub>, 0.8 mg ml<sup>-1</sup> bovine serum albumin (BSA, New  
132 England Biolabs GmbH), 0.2 mM of each dNTP (Bioline, Luckenwalde, Germany), 0.2 µM of each  
133 primer, and 0.5 units of Phusion Hot Start Flex DNA polymerase (New England Biolabs GmbH).

134 Thermal cycles were carried out in a Mastercycler pro thermal cycler (Eppendorf, Hamburg,  
135 Germany) with the following program: 94 C for 1 min followed by 35 cycles of 94 C for 30 s, 52 C  
136 for 30 s, and 68 C for 30 s; and a final step of 68 C for 10 min. The amplicons were visualized in an  
137 electrophoresis agarose gel and volumes of individual PCR products between 6 and 12  $\mu$ l were  
138 pooled based on band intensity (Duhamel et al., 2019). The DNA pool was purified with the  
139 Zymoclean TM Gel DNA Recovery Kit (Zymo Research, Freiburg, Germany), quantified with a  
140 Qubit Fluorometer (Thermo Fisher Scientific), and paired-end sequenced by Eurofins Genomics  
141 GmbH (Ebersberg, Germany) with the Illumina MiSeq platform, using the MiSeq Reagent Kit v3  
142 (Illumina Inc., San Diego, CA, USA).

143 For amplification of the oomycete *cox2* gene, primers Cox2-F and Cox2-RC4 (Choi et al., 2015;  
144 Hudspeth et al., 2000) were used (Table S4). PCR reactions were done as described above, but with  
145 the polymerase MangoTaq (Bioline, Luckenwalde, Germany), using the following thermal  
146 conditions: 94 C for 4 min followed by 36 cycles of 94 C for 40 s, 53 °C for 20 s, and 72 C for 60 s;  
147 and a final step of 72 C for 4 min. Library preparation and sequencing with the MiSeq platform was  
148 performed by LGC Genomics GmbH (Berlin, Germany).

149 The sequence data generated in this study has been deposited in the NCBI Sequence Read Archive  
150 under BioProject number PRJNA593383.

151

## 152 *Sequence processing*

153 Sequence reads from the fungal ITS1 region and the oomycete *cox2* gene were processed using the  
154 DADA2 pipeline (Callahan et al., 2016) for quality filtering, dereplication, removal of chimeric  
155 sequences, and grouping of reads into exact amplicon sequence variants (ASVs; Callahan et al.,  
156 2017). In the case of the ITS1, the process included the merging of paired forward and reverse reads  
157 after the dereplication step. The paired-end merging was omitted in the case of *cox2* sequences  
158 given the frequent lack of overlapping regions between forward and reverse reads, owing to the  
159 length of the amplicon. In this case, only forward reads were processed due to their overall higher

160 quality respect to the reverse reads. The code used to process both paired-end and single direction  
161 sequences is available online at [https://github.com/jgmv/MiSeq\\_process](https://github.com/jgmv/MiSeq_process).  
162 Taxonomic annotation of ASV sequences was done with the Naïve Bayesian Classifier tool (Wang  
163 et al., 2007) available in MOTHUR v1.39.5 (Schloss et al., 2009). For the fungal ITS1, annotation was  
164 achieved by comparing sequences against the UNITE database of fungal ITS sequences (Kõljalg et  
165 al., 2005). Because no similar database is available for the oomycete *cox2* gene, in this case we used  
166 an *ad hoc* built reference sequence data set with all oomycete *cox2* sequences available in NCBI  
167 GenBank as of July 26<sup>th</sup>, 2019.

168

### 169 *Statistical analyses*

170 All statistical analyses were carried out in R v3.6.1 (R Core Team, 2019). Both the ITS1 and *cox2*  
171 data sets were processed independently, but using the same procedures. First, ASVs represented  
172 globally by less than five reads were discarded, as well as nine samples from the *cox2* dataset that  
173 contained no reads. Sampling coverage was assessed by the construction of rarefaction curves per  
174 sample, using functions in R package VEGAN v2.5-4 (Oksanen et al., 2019). These showed marked  
175 differences in read abundances and ASV richness, although in most cases saturation in the ASV  
176 accumulations indicated appropriate coverage of samples' richness (Fig. S1). We relied on a mixture  
177 model to normalize reads and account for differences in library size and biological variability, using  
178 the variance stabilization method with package DESEQ v1.35.1 (Anders and Huber, 2010; Love et  
179 al., 2014). This method has shown to outperform rarefying based-normalization because it does not  
180 require samples or species with few reads to be discarded and does not decrease the statistical  
181 power in analyses (McMurdie and Holmes, 2014).

182 Diversity and community structure analyses relied on functions in package VEGAN. ASV richness  
183 and diversity, based on the Shannon diversity index ( $H'$ ) and represented as effective species  
184 numbers ( $e^{H'}$ ; Jost, 2006), were calculated per sample using the non-normalized datasets. Significant  
185 differences in reads abundance, richness, and diversity across plant species and compartments were



186 assessed by means of the Kruskal-Wallis rank sum tests. In the case of comparisons of between  
187 sampling dates, paired Wilcoxon rank sum tests were used. Kruskal-Wallis tests with Bonferroni  
188 adjustment of  $P$  values to account for multiple comparisons were applied to assess significant  
189 variations in the abundance of individual taxa across soil/root compartments. Pearson's correlation  
190 coefficient ( $r$ ) was used to assess relationships between read numbers and richness or diversity  
191 values per sample.

192 To investigate differences in community composition across plant species, soil/root compartment,  
193 and sampling date, we calculated Bray-Curtis dissimilarities among samples in the normalized  
194 datasets, and visualized them using unconstrained principal coordinates analysis (PCoA)  
195 ordinations. To further assess the contribution of variables to community variation, constrained  
196 distance-based redundancy analysis (db-RDA) was applied, using plant species, soil/root  
197 compartment, and sampling date as constraining factors.

198 We investigated the specific association of fungal and oomycete ASVs with rhizospheric and plant  
199 compartments by assessing their enrichment or depletion respect to bulk soil, following a  
200 differential abundance analysis adapted from that described in Edwards et al. (2015). For this, data  
201 from compartments bulk soil and root zone soil were combined, and the fold change abundance of  
202 individual ASVs in rhizosphere, root, and endosphere compartments vs. soil were calculated based  
203 on the coefficients of fitted generalized linear models (GLM) of abundance data. The non-  
204 normalized data set with read counts was used to build the models based on a negative binomial  
205 regression, with read numbers across samples accounted for by including them as a model  
206 parameter (i.e.  $abundance \sim reads + compartment$ ). Differential abundance was calculated based on  
207 the GLM coefficients for the rhizosphere, root, and endosphere compartments, representing fold  
208 change respect to soil, and tested by the likelihood ratio test with an adjusted  $P$  value cutoff of 0.01.  
209 Positive coefficient values indicate enrichment of and ASV's abundance in a compartment respect  
210 to its abundance in soil, whereas negative values indicate depletion (Edwards et al., 2015).

211 All the data sets and the BASH and R code used for the data pre-processing and statistical analyses  
212 has been made available online at  
213 [https://github.com/jgmv/Brassicaceae\\_fungal\\_and\\_oomycete\\_root\\_microbiome](https://github.com/jgmv/Brassicaceae_fungal_and_oomycete_root_microbiome).

214

## 215 **Results**

216 A total of 7,567,288 sequence reads representing 4,289 fungal ASVs were retained in the fungal ITS  
217 dataset after quality filtering and discarding rare ASVs. For the oomycete *cox2* dataset, 4,105,321  
218 reads representing 951 ASVs were kept. In the case of *cox2* data, nine samples from the root and  
219 endosphere compartments of *A. thaliana* and *D. verna* were dropped due to absence of reads  
220 (Table 1). Despite attempts to normalize the quantity of amplified DNA prior to MiSeq sequencing,  
221 there was a strong effect of plant compartment on the number of ITS reads per sample, with roots  
222 and endosphere showing significantly lower read numbers (Kruskal-Wallis test,  $H = 50.8$ ,  $df = 4$ ,  
223  $P < 0.01$ ; Table 1, Fig. S2) than soil samples. This effect was not significant in the case of *cox2*  
224 reads, neither had plant species a strong effect on either ITS and *cox2* read numbers (Table 1,  
225 Fig. S2). Likewise, plant compartment, but not plant species, affected fungal ( $H = 98.4$ ,  $df = 4$ ,  
226  $P < 0.01$ ) and oomycete ( $H = 80.5$ ,  $df = 4$ ,  $P < 0.01$ ) ASV richness. Date of sampling had a  
227 significant effect on the number of reads (paired Wilcoxon test,  $V = 1752$ ,  $P < 0.001$ ) and ASV  
228 richness ( $V = 1687.5$ ,  $P < 0.001$ ) per sample in the fungal ITS dataset, but not in the oomycete  
229 dataset (Fig. S2).

230 Shannon diversity was less affected by read abundances than ASV richness ( $r = 0.01$ ,  $P = 0.9$  vs.  
231  $r = 0.26$ ,  $P = 0.003$  in the ITS dataset;  $r = 0.12$ ,  $P = 0.2$  vs.  $r = 0.17$ ,  $P = 0.06$  in the *cox2* dataset),  
232 and hence was further used to evaluate diversity patterns across factors (Fig. 1). Again, the soil/root  
233 compartment significantly affected diversity in both fungal ( $H = 81.1$ ,  $df = 4$ ,  $P < 0.01$ ) and  
234 oomycete ( $H = 81.2$ ,  $df = 4$ ,  $P < 0.01$ ) communities, most markedly by clearly lower numbers of  
235 effective ASVs in the root and endosphere as compared to soil compartments (Fig. 1). Plant species  
236 and sampling date did not show a relationship with fungal or oomycete diversity, although samples

237 collected in April appeared to have larger variation in diversity values than those collected in  
238 February (Fig. 1).

239 The ordination analysis of fungal and oomycete community structures showed similar patterns to  
240 those of diversity, with samples from soil and root compartments forming two main, separate  
241 clusters (Fig. 2A,C). db-RDA ordination of fungal communities explained a significant 26.2 % of  
242 overall community variation (pseudo- $F_{7,124} = 6.3$ ,  $P = 0.001$ ). Of this variation, the largest fraction  
243 was related to differences in soil/root compartment (Fig. 2B), with all soil compartments forming a  
244 compact cluster well separated from root and endosphere samples, which also tended to cluster  
245 separately from each other (Fig. 2A). A similar pattern was found for oomycetes, although in this  
246 case the db-RDA only explained an 8.5 % of community variation (pseudo- $F_{7,115} = 1.5$ ,  $P = 0.001$ )  
247 and, even though compartment was again the most explanatory factor (Fig. 2E), there was a lesser  
248 definition of the db-RDA clusters (Fig. 2D). Separate db-RDA ordinations were calculated for the  
249 different soil/root compartments to assess the effects of plant species and sampling date on different  
250 fractions of the microbial communities (Figs. 2C,F). These showed in all cases that plant species  
251 explained a larger fraction of community variation than sampling date, although this was relatively  
252 low and did not change markedly across compartments.

253 Most fungal ASVs belonged in the Ascomycota (48.5 %), followed by the Basidiomycota (20 %),  
254 the Glomeromycota (2.2 %), and the Mortierellomycota (2.2 %); and were classified into 86 orders,  
255 plus 17 and 3 taxa unclassified or with uncertain classification (*Incertae sedis*) at the order level  
256 (Table S1). The most abundant orders showed a significant variation in their relative abundance  
257 across soil/root compartments (Table S2), which was consistent across plant species (Fig. 3A). This  
258 was most evident by an increase in the representation of ASVs in the Pleosporales ( $H = 91.7$ ,  $df = 4$ ,  
259  $P_{adj} < 0.001$ ), Helotiales ( $H = 85.3$ ,  $df = 4$ ,  $P_{adj} < 0.001$ ), Olpidiales ( $H = 32.6$ ,  $df = 4$ ,  $P_{adj} < 0.001$ ),  
260 and Cantharellales ( $H = 88$ ,  $df = 4$ ,  $P_{adj} < 0.001$ ) in the root compartments; and by the opposite  
261 pattern in the Chaetothyriales ( $H = 103.6$ ,  $df = 4$ ,  $P_{adj} < 0.001$ ) and Mortierellales ( $H = 99.4$ ,  $df = 4$ ,  
262  $P_{adj} < 0.001$ ), as well as in the unclassified Fungi ( $H = 99.3$ ,  $df = 4$ ,  $P_{adj} < 0.001$ ). In oomycetes,

263 most ASVs remained unclassified at all taxonomic levels (Fig. 3A, Table S3). Those that could be  
264 assigned to an order were distributed across 5 orders, with the Pythiales comprising the largest  
265 number of ASVs (34.5 %), followed by far by the Peronosporales (5.3 %) and the Saprolegniales  
266 (3 %). In this case, no evident patterns in the distribution of the orders abundances across soil/root  
267 compartments was found.

268 The differential abundance analyses of ASVs showed that fungi were much more depleted than  
269 enriched in rhizosphere and root compartments respect to soil, with 2160 depleted vs. 869 enriched  
270 ASVs, whereas oomycetes appeared to be enriched or depleted at similar rates (Fig. 3B; Table S3).  
271 In both fungi and oomycetes, enrichment of ASVs occurred mostly in the rhizosphere. On the  
272 contrary, most ASVs were depleted in all compartments, although others were exclusively depleted  
273 in both root compartments (Fig. 3B). In all cases, several ASVs appeared to be exclusively enriched  
274 or depleted at specific compartments (Fig. 3B). In fungi, the root-enriched ASVs belonged mostly  
275 to the Helotiales, which increased their representation respect to soil both in the root surface and in  
276 the endosphere (Fig. 3C; Table 2). They were followed by the Pleosporales and Pezizales (solely  
277 represented by the genus *Tuber*), which became enriched in the outer and inner root, respectively  
278 (Fig. 3C; Table 2). In oomycetes, the major enrichment in roots included *Pythium rostratifingens*,  
279 *Phytophthora citricola*, and *Saprolegnia ferax*, as representatives of the Pythiales, Peronosporales,  
280 and Saprolegniales (Fig. 3C; Table 2). The ASVs depleted in rhizosphere and plant compartments  
281 belonged to multiple fungal and oomycete orders (Fig. 3C), somewhat mirroring the overall  
282 distribution of orders found for both groups (Fig. 3A).

283

## 284 **Discussion**

285 The fungal and oomycete communities in roots of three brassicaceous plant species and their  
286 associated soil were barely affected by host identity and a time of sampling spanning two months.  
287 Instead, the major driver of community differences was the soil/root compartment, which  
288 determined differences in community diversity and structure between the microbiota developing in

289 soil and different parts of the roots. These results suggest that the interface between soil and roots  
290 exerts a strong barrier to root colonization, but also that those fungi and oomycetes able to surpass  
291 the root boundaries are generalist groups without strong host preferences.

292 The remarkable differences between soil and root-associated fungal and oomycete communities  
293 described here have been already reported for multiple microbial groups and plant lineages. Studies  
294 on several plant species have found compartments, ranging from root-associated soil to the root  
295 endosphere, to be the strongest determinants of community variation, following patterns similar to  
296 those found here, both in fungi (Almarío et al., 2017; Coleman-Derr et al., 2016; Durán et al., 2018;  
297 Martínez-Diz et al., 2019; Thiergart et al., 2019) and oomycetes (Durán et al., 2018; Thiergart et al.,  
298 2019), but also in other microbial groups like protists (Sapp et al., 2018) and bacteria (Bulgarelli et  
299 al., 2012; Coleman-Derr et al., 2016; Durán et al., 2018; Edwards et al., 2015; Lundberg et al.,  
300 2012; Thiergart et al., 2019). Altogether, there is strong evidence that the root surface exerts a  
301 control on the entry of soil microorganisms into the root tissues. This control has been proposed to  
302 depend on two main processes: an enrichment from soil towards the root tissues of soil microbes  
303 with root-colonizing traits, starting already at a certain distance from the rhizoplane probably in  
304 response to root exudates; and an exclusion of the largest fraction of soil microorganisms, mostly  
305 taking place at the rhizoplane level (Edwards et al., 2015; Heijden and Schlaeppi, 2015). This  
306 model of microbial recruitment, proposed for bacteria, was only partly met by our results, and it  
307 varied between fungi and oomycetes. Here, the blockade of microbial entry into the roots was  
308 already observed away from the root surface, since most ASVs were jointly depleted from the  
309 rhizosphere toward the inner root tissues, and appeared to be unspecific because most orders  
310 decreased proportionally to their overall abundance. Bacteria that respond to root exudates by  
311 locally increasing their populations and activating antimicrobial functions have been shown to play  
312 a role in the suppression of fungal root pathogens at the rhizosphere level (Chapelle et al., 2016;  
313 Leveau et al., 2010), a process that could be responsible for unspecifically inhibiting growth of  
314 other fungi and oomycetes (Durán et al., 2018). This could also explain the lack of an important

315 distance enrichment of ASVs in the rhizosphere, as has been observed for bacteria (Edwards et al.,  
316 2015). In addition, few or no enriched ASVs were shared among the rhizosphere, root, and  
317 endosphere compartments, indicating that each of these likely represent specialized niches for  
318 different taxa.

319 Plant species did not affect considerably community structure, neither from fungi nor from  
320 oomycetes. Neither this effect increased in root compartments respect to the surrounding soil, where  
321 the influence of species-specific chemistry, defense responses, or microbial recognition, would  
322 expectably lead the processes of microbial enrichment and depletion described above. However,  
323 this finding is in line with previous reports of a limited host specificity of root-associated microbes  
324 (Bulgarelli et al., 2012; Colin et al., 2017; Dombrowski et al., 2016; Glynou et al., 2018b; Schlaeppi  
325 et al., 2014; Thiergart et al., 2019; Wagner et al., 2016; Zarraonaindia et al., 2015). This is at least  
326 true in related plant species, such as those investigated in our study, because phylogenetically  
327 distant hosts harbor clearly distinguishable microbial communities (U'Ren et al., 2019) likely due to  
328 major differences in cellular physiology and chemistry. The ability of endophytes to colonize  
329 multiple hosts appears to be frequent among non-pathogenic symbionts (Pölme et al., 2018), in  
330 stark contrast to the high host specialization found throughout the pathogenic lifestyle (Thines,  
331 2019). The loose host preferences of endophytes may enable them to persist in and disseminate  
332 across habitats with diverse communities of potential plant hosts.

333 As with host identity, the sampling time did not affect significantly the structure of microbial  
334 communities in soil or roots. Particularly in roots, the temporal effect would have been ascribed to  
335 two processes: the normal seasonal variation in soil microbial communities, which would have been  
336 detectable in plant-free soil samples; and the physiological changes in root tissues upon  
337 phenological stages, which would likely have strongest effects in root compartments. Changes in  
338 soil microbiota have been shown to occur over seasons, although they are smaller than those  
339 triggered by other ecological factors, such as habitat type or spatial distance, and they are more  
340 evident over longer periods of time than those considered in this study (He et al., 2017). Regarding

341 phenotype-driven modifications of the plant microbiome, Dombrowski et al, (2016) showed that  
342 non-flowering wild types of the brassiaceous species *Arabidopsis thaliana* assembled bacterial root  
343 communities indistinguishable from those of flowering mutants, and concluded that the root  
344 microbiota is robust to changes following its established in earlier stages of plant development. Our  
345 results reinforce this conclusion, expanding it to eukaryotic members of the root microbiome.  
346 A broad phylogenetic diversity of fungi were found associated with roots of the three Brassicaceae  
347 species studied, reflecting the taxonomic fungal diversity in the surrounding soil. As this study,  
348 previous works have repeatedly shown a consistent dominance of root fungal endophytic  
349 communities by members of the Pleosporales, Hypocreales, and Helotiales (Bonito et al., 2014;  
350 Glynou et al., 2018a, 2018b; Keim et al., 2014; Knapp et al., 2012), which likely occupy  
351 complementary niches within roots (Kia et al., 2019). However, our new data suggest that  
352 prevalence of hypocrealean fungi might depend on their soil abundance, as they were not  
353 importantly enriched in root tissues. The Helotiales were particularly enriched in the root and  
354 endosphere compartments, in line with previous studies and the known association of this order  
355 with plant roots, where they might be involved in assisting host nutrition, as has been shown in  
356 some instances (Almario et al., 2017; Johnston et al., 2019). In contrast to fungi, enrichment  
357 patterns in oomycetes were less clear, and involved groups of well-known root pathogens like  
358 *Pythium* and *Phytophthora*. In this case, most ASVs remained unclassified. Even though the *cox2*  
359 gene has been shown adequate for species identification and phylogenetics within the oomycetes  
360 (Choi et al., 2015), the low diversity of *cox2* sequences represented in public repositories presently  
361 hinders its application as barcoding marker.  
362 Our sample processing yielded an overall lower sequencing depth in the root than in soil samples,  
363 and that was particularly low in the endosphere. These differences in sampling coverage across  
364 compartments may have influenced to some extent the results on diversity and community structure,  
365 reducing resolution in ASV detection. These differences likely reflect the low amount of fungal and  
366 oomycete DNA, and hence biomass, within plant tissues. This problem when amplifying

367 endophytic DNA has been overcome elsewhere by the application of nested PCR prior to  
368 sequencing (Eusemann et al., 2016; Unterseher et al., 2016), although we opted to avoid this  
369 procedure due to potential risks of distorting relative abundances and community structures detected  
370 (Yu et al., 2015). Nevertheless, the diversity and community patterns detected are consistent with  
371 those obtained in similar studies, thus indicating a valid biological signal in our results.  
372 In conclusion, our results show that fungi assemble more diverse and complex communities than  
373 oomycetes in plant roots and their associated soil. However, microbial communities of both groups  
374 are similarly structured across rhizocompartments, and relatively lowly affected by plant identity  
375 and phenology of annual Brassicaceae sharing the same habitat. The enrichment of specific lineages  
376 in roots, particularly in fungi, suggests a level of specialization towards the asymptomatic  
377 colonization of below-ground plant tissues, hinting symbiotic lifestyles which could be relevant to  
378 host's fitness and health. The ecological meaning of these interactions, however, remains unknown  
379 and warrants further study.

380

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386

### 387 **Author Contribution**

388 M.T. designed the study. B.N. and M.T. collected the samples. B.N. processed the samples and  
389 prepared the DNA extracts. J.G.M.-V. and B.N. prepared the libraries for Illumina MiSeq  
390 sequencing. M.T. and J.G.M.-V. contributed materials and reagents. J.G.M.-V. analyzed the data and  
391 prepared a first draft of the manuscript, with input from B.N. and M.T. All authors contributed to  
392 the final version of the manuscript.



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395 **Table 1.** Distribution of numbers of reads and amplicon sequence variants (ASVs) richness across fungal and oomycete samples.

		Fungi						Oomycota					
		February			April			February			April		
		n	Reads / plant <sup>1</sup>	Richness	n	Reads / plant	Richness	n	Reads / plant	Richness	n	Reads / plant	Richness
<i>A. thaliana</i>	Bare soil	3	57121 ± 9202	686	3	97170 ± 54434	759	3	41594 ± 21817	73	3	10954 ± 7898	59
	Root soil	3	69249 ± 1304	743	3	69060 ± 28821	847	3	28300 ± 10051	64	3	48440 ± 35566	58
	Rhizosphere	10	35093 ± 22781	999	10	94449 ± 78228	1522	10	33911 ± 31632	192	10	34644 ± 26163	214
	Root	10	21189 ± 29870	214	10	130423 ± 176687	313	10	33190 ± 33411	44	9	56005 ± 37747	88
	Endosphere	10	14630 ± 43466	89	10	37609 ± 99336	128	8	50724 ± 92048	20	8	33853 ± 53642	20
<i>C. hirsuta</i>	Bare soil	3	36908 ± 25218	501	3	73449 ± 19219	870	3	38093 ± 24432	67	3	7636 ± 5203	46
	Root soil	3	62464 ± 14017	737	3	113586 ± 79217	789	3	25880 ± 8770	56	3	22933 ± 23163	50
	Rhizosphere	3	55227 ± 3139	694	3	134748 ± 101309	762	3	21413 ± 17630	70	3	19186 ± 10021	69
	Root	3	44161 ± 50344	74	3	190322 ± 133137	126	3	80482 ± 138248	16	3	50206 ± 47608	13
	Endosphere	3	523 ± 282	31	3	4041 ± 6329	43	3	39416 ± 68215	7	3	4180 ± 5795	6
<i>D. verna</i>	Bare soil	3	41529 ± 18834	606	3	60902 ± 4129	810	3	25264 ± 23975	67	3	46958 ± 54960	61
	Root soil	3	52975 ± 17889	573	3	84638 ± 11754	939	3	31846 ± 22576	51	3	15610 ± 18422	57
	Rhizosphere	3	69498 ± 2484	710	3	70873 ± 26902	870	3	19708 ± 18113	62	3	20748 ± 20390	70
	Root	3	4472 ± 4044	40	3	9982 ± 13726	59	3	6103 ± 10295	7	3	30661 ± 39981	20
	Endosphere	3	439 ± 121	16	3	7780 ± 11554	30	1	285 ± 0	1	1	93 ± 0	1

396 <sup>1</sup> Values represent the mean number of reads per plant ± standard deviation.

397 **Table 2.** Fungal and oomycete amplicon sequence variants (ASVs) enriched in the endosphere  
 398 compartment.

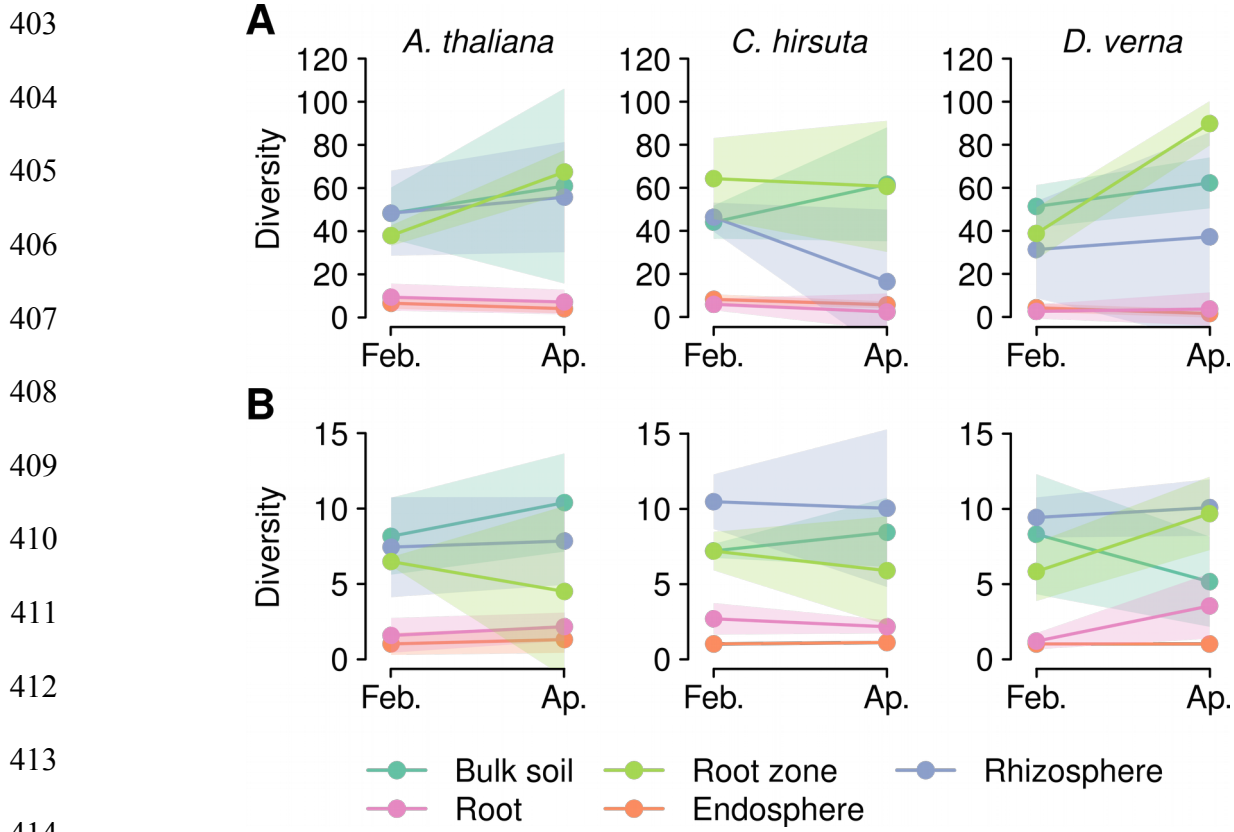
	ASV	Compartment <sup>1</sup>	Host <sup>2</sup>		
			<i>A.</i> <i>thaliana</i>	<i>C.</i> <i>hirsuta</i>	<i>D.</i> <i>verna</i>
Fungi					
<i>Calycina</i> sp. (Helotiales)	FU00009	e	+	+	+
<i>Tuber</i> sp. (Pezizales)	FU00160	e,r	+	+	+
<i>Kabatiella</i> sp. (Dothideales)	FU00212	e,r	+	-	+
<i>Paraphoma</i> sp. (Pleosporales)	FU00277	e	+	+	+
unclassified Helotiales	FU00433	e,r	+	-	+
unclassified Helotiales	FU00607	e	+	-	-
unclassified Coniochaetales	FU00766	e,r	+	+	+
unclassified Sebaciales	FU00802	e	+	-	-
<i>Athelia</i> sp. (Atheliales)	FU00851	e,r	+	+	+
<i>Apiotrichum xylopinii</i> (Trichosporonales)	FU00893	e	+	-	+
<i>Aspergillus</i> sp. (Eurotiales)	FU00930	e,r	+	+	+
unclassified Sordariomycetes	FU00953	e	+	+	+
<i>Stemphylium</i> sp. (Pleosporales)	FU00961	e,r	+	-	-
unclassified Dothideomycetes	FU00966	e	+	+	+
<i>Calycina ellisii</i> (Helotiales)	FU01001	e	+	-	-
unclassified Ceratobasidiaceae (Cantharellales)	FU01006	e	+	+	-
unclassified Sebaciales	FU01030	e	+	-	-
<i>Dactylonectria</i> sp. (Hypocreales)	FU01089	e	+	+	+
unclassified Hydnodontaceae (Trechisporales)	FU01292	e	+	-	-
unclassified Rutstroemiaceae (Helotiales)	FU01388	e	-	+	-
<i>Tetracladium marchalianum</i> (Helotiales)	FU01433	e	-	-	+
unclassified Nectriaceae (Hypocreales)	FU01515	e	-	-	+
unclassified Helotiales	FU01572	e	-	+	-
unclassified Thelebolaceae (Thelebolales)	FU01626	e	+	-	-
<i>Tuber borchii</i> (Pezizales)	FU01638	e	-	+	-
unclassified Helotiales	FU01696	e	+	-	-
unclassified Helotiales	FU01775	e	+	-	-
<i>Plectosphaerella cucumerina</i> (Glomerellales)	FU01776	e	-	+	-
<i>Tetracladium marchalianum</i> (Helotiales)	FU01777	e	-	-	+
<i>Articulospora</i> sp. (Helotiales)	FU01797	e	+	-	-
<i>Tuber borchii</i> (Pezizales)	FU01828	e	+	-	-
<i>Tuber oligospermum</i> (Pezizales)	FU01829	e	-	-	+
<i>Rhizodermea</i> sp. (Helotiales)	FU01870	e	-	-	+
unclassified Fungi	FU01943	e	-	-	+

<i>Thanatephorus cucumeris</i> (Cantharellales)	FU01967	e	-	+	-
<i>Tetracladium marchalianum</i> (Helotiales)	FU02008	e	+	-	-
<i>Tuber oligospermum</i> (Pezizales)	FU02049	e	+	-	-
<i>Apiotrichum gracile</i> (Trichosporonales)	FU02050	e	+	-	-
<i>Tetracladium marchalianum</i> (Helotiales)	FU02071	e	+	-	-
<i>Tuber oligospermum</i> (Pezizales)	FU02240	e	-	-	+
unclassified Capnodiales	FU02499	e	+	-	-
<b>Oomycota</b>					
unclassified Oomycetes	OO00004	e,r	+	+	+
unclassified Oomycetes	OO00005	e	+	+	-
unclassified Oomycetes	OO00024	e,r	+	-	-
<i>Pythium rostratiformis</i> (Pythiales)	OO00028	e	+	-	+
unclassified Saprolegniaceae (Saprolegniales)	OO00047	e	+	+	-
<i>Pythium rostratiformis</i> (Pythiales)	OO00048	e,r	+	-	+
unclassified Oomycetes	OO00063	e	+	-	-
unclassified Oomycetes	OO00066	e	+	-	-
unclassified Oomycetes	OO00067	e	-	+	-
<i>Phytophthora citricola</i> (Peronosporales)	OO00071	e,r	+	-	-
unclassified Oomycetes	OO00075	e	+	-	-
unclassified Oomycetes	OO00088	e	+	-	-
<i>Saprolegnia ferax</i> (Saprolegniales)	OO00117	e	-	+	-
unclassified Oomycetes	OO00122	e	+	-	-
<i>Saprolegnia ferax</i> (Saprolegniales)	OO00142	e	+	-	-
unclassified Oomycetes	OO00154	e	+	+	+
unclassified Oomycetes	OO00167	e	+	-	-
<i>Phytophthora citricola</i> (Peronosporales)	OO00195	e	+	-	-
<i>Saprolegnia ferax</i> (Saprolegniales)	OO00317	e	+	-	-
unclassified Oomycetes	OO00478	e	+	-	-
unclassified Oomycetes	OO00485	e	-	-	+
unclassified Oomycetes	OO00679	e	+	-	-
unclassified Oomycetes	OO00689	e,r	+	+	-
unclassified Pythiales	OO00755	e	+	-	-
unclassified Oomycetes	OO00793	e	+	-	-
<i>Saprolegnia ferax</i> (Saprolegniales)	OO00801	e	-	+	-
unclassified Peronosporales	OO00836	e	+	-	-
unclassified Peronosporaceae (Peronosporales)	OO00916	e	-	+	-
unclassified Oomycetes	OO00920	e	+	-	-

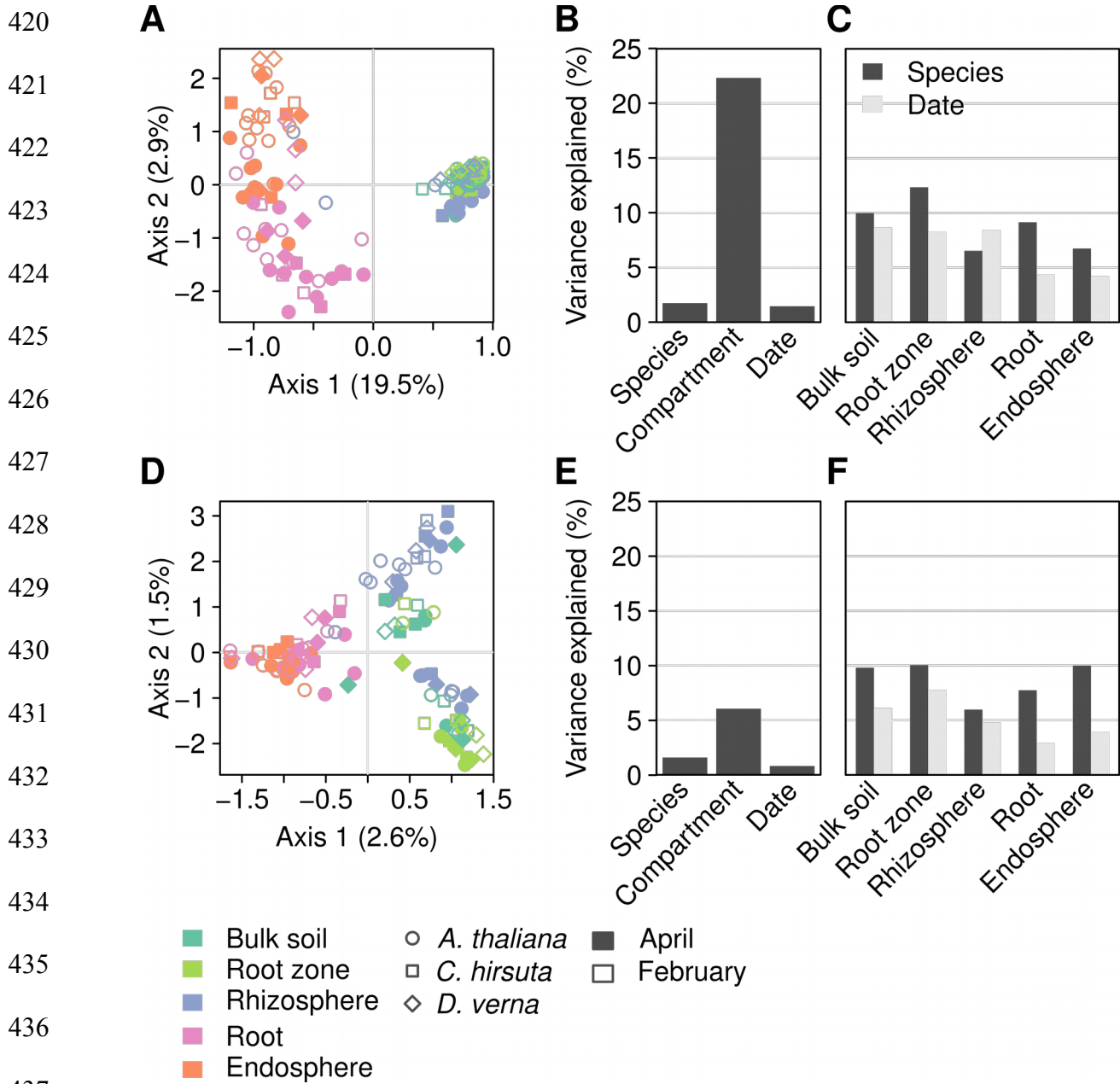
399 <sup>1</sup> Whether the ASVs was detected in non-sterilized roots.Plant compartment where the ASVs were  
400 enriched: r, root; e, endosphere.

401 <sup>2</sup> Hosts in which the ASVs were detected.

402

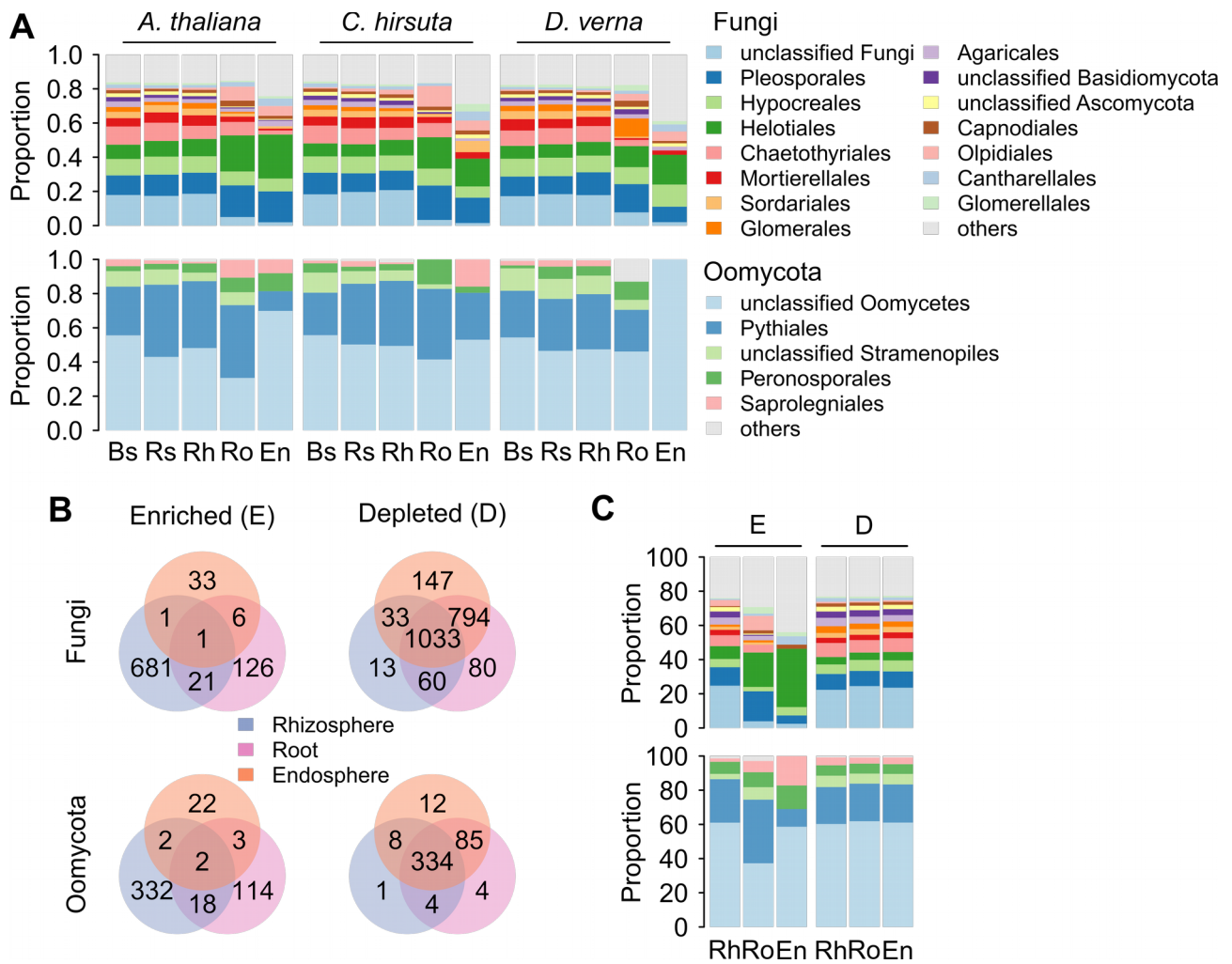






438 **Figure 2.** Effect of host identity, date of sampling, and soil/root compartment on the structure of  
 439 fungal (A – C) and oomycete (D – F) communities. Plots in A and C show db-RDA ordinations  
 440 based on Bray-Curtis dissimilarities, constrained by host, date, and compartment. B and D show the  
 441 variance explained in db-RDAs by each of the three factors, whereas C and F show the variance  
 442 explained by host species and date in subsets of data for each soil/root compartment.

443



444 **Figure 3.** Taxonomic structure of fungal and oomycete communities and variation of ASV's  
 445 abundance across soil/root compartments. Bar plots in **A** show the distribution of fungal (top) and  
 446 oomycetes (bottom) orders across host species and soil/root compartments. Venn diagrams in **B**  
 447 show the numbers of differentially enriched or depleted ASVs in rhizocompartments as compared to  
 448 bulk soil. **C** shows the proportion of enriched (**E**) and depleted (**D**) ASVs in different  
 449 rhizocompartments within the main fungal and oomycete orders. Abbreviations: **Bs**, bulk soil; **Rs**,  
 450 root zone soil; **Rh**, rhizosphere; **Ro**, root; **En**, endosphere.

451

452 **Supplementary information**

453 **Table S1.** Taxonomic classification of fungal and oomycete ASVs.

454 **Table S2.** Variation across soil/root compartments in the abundance of fungal and oomycete orders.

455 **Table S3.** Significant ( $P < 0.01$ ) differential abundances of fungal and oomycete ASVs across  
456 compartments.

457 **Table S4.** Primers used in this study.

458 **Figure S1.** Rarefaction curves of ASVs accumulation with sequence reads, for the fungal ITS (**A**)  
459 and the oomycete *cox2* gene (**B**) datasets.

460 **Figure S2.** Reads (**A**) and ASVs richness (**B**) values obtained by Illumina MiSeq sequencing across  
461 the factors considered in this study. Box-and-whisker plots summarize the distribution of each  
462 measurement (median, interquartile range, and range) per factor, and points show individual values  
463 per sample.