

<sup>1</sup> **The effect of *Ascophyllum nodosum* extracts on tomato  
2 and pepper plant productivity and their associated  
3 fungal and bacterial communities.**

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<sup>7</sup> The abstract will be written here

<sup>8</sup> *Keywords:* Stella Marris, 16S, ITS, microbial diversity, Illumina MiSeq

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**9 INTRODUCTION**

10 Liquid extracts of marine macroalga are used as biostimulants in agriculture. These extracts con-  
11 tain phytohormones that can influence physiological processes even at very low concentrations  
12 Craigie (2011). Stella Maris® is derived from fresh *Ascophyllum nodosum* harvested from the  
13 nutrient-laden waters of the North Atlantic off the Eastern Coast of Canada.

14

15 The aim of this project was to develop a better understanding of the effects of *A. nodosum* extracts  
16 on plant growth. We tested the effect of these extract on two commonly used plants (Tomato -  
17 *Solanum lycopersicum* and Pepper - *Capsicum annuum*) using different measures of productivity. In  
18 addition, we tested how the bacterial and fungal communities responded to the addition of *A.  
19 nodosum* extracts.

20

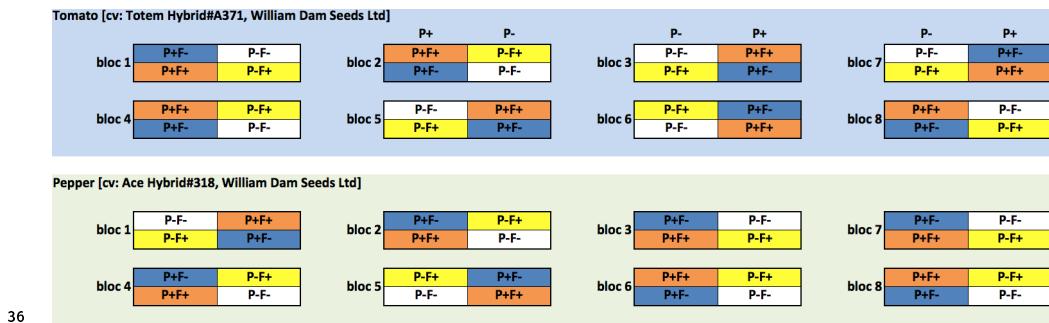
**21 MATERIAL AND METHOD**

**22 Study design**

23 Two greenhouse experiment were set up in large trays (60x30x18 cm) in November (tomato [cv:  
24 Totem Hybrid#A371, William Dam Seeds Ltd]) and December (Pepper [cv: Ace Hybrid#318,  
25 William Dam Seeds Ltd]) 2015. Soil was collected form an agricultural field under organic regime  
26 at the IRDA research station in St-Bruno (Qc, Canada) on October 7th 2015 (loam sandy soil, 15 cm  
27 top layer collected). Soil characteristics (four samples) were measured by AgriDirect (Longueuil,  
28 Qc, Canada) to determine...

29

30 For each species tested (Tomato - *Solanum lycopersicum*, Pepper - *Capsicum annuum*), a random-  
31 ized split block design (Figure 1) was used with four trays set up per block (eight blocks). Half  
32 of the trays were fertilized (fertilization treatment), as described below. Half of the trays were  
33 also planted with four replicate plants each, while the other trays were left bare. This allowed  
34 comparing the fungal and bacteria soil communities with respect to the fertilization and planting  
35 treatment.



36

### 37 **Figure 1: experimental design**

38

39 Tomato plants were fertilized using multipurpose organic fertilizer (pure hen manure, 18 g per  
 40 tray repeated every 4 weeks, 5-3-2) from Acti-sol (Notre-Dame-du-Bon-Conseil, Qc, Canada) in  
 41 addition to Stella Maris® (3.5 ml per 1L, each tray received 250 ml, repeated every 2 weeks) for  
 42 the duration of the experiment. Stella Maris® is a registered trademark from Acadian Seaplants  
 43 Ltd. (Darmouth, NS, Canada). It is primarily composed of *Ascophyllum nodosum* seaweed and is  
 44 advertised as a natural activator of the crops' own growth and defense mechanisms to improve  
 45 root growth and resist temperature, drought, and salinity stress in order to maximize yield and  
 46 crop qualities (Acadian Seaplants Ltd. 2018). Pepper plants were fertilized using solely Stella  
 47 Maris (3.5 ml per 1L, each tray received 250 ml, repeated every 2 weeks) for the duration of the  
 48 experiment.

49

50 Thrips were managed with *Neoseiulus cucumeris* (syn. *Amblyseius cucumeris*) (100 bags), Fungus  
 51 gnat and thrips were also controlled using predatory mite *Gaeolaelaps gillespiei* (1L). Plants were  
 52 treated once a week with Oidium Milstop.

53

54

### 55 *Plant productivity*

56 At the end of the experiment, plant productivity was assessed by measuring four different traits  
 57 (fruit number, average fruit weight, shoots fresh weight, roots fresh weight) on three plants chosen  
 58 randomly per tray (for each treatment [fertilization/control], species [tomato/pepper] and block  
 59 [eight blocks]) for a total of 96 samples. In addition, both shoots and roots were dried in a 70  
 60 degrees drying oven, and dry weights were measured after 48 hours. Together, these traits are

61 expected to represent well the plant overall productivity.

62

63

64 *Sample preparation, DNA extraction and High throughput sequencing*

65 We sampled both the microbial and fungal communities from soil and root samples. Soil DNA  
66 was extracted using XXX DNA isolation kit with YYY g of soil. Roots were first washed with  
67 sterile water and DNA was extracted using XXX DNA isolation kit with YYY g of root samples.  
68 Amplicon sequencing targeting 16S rRNA gene (bacteria) and ITS (fungi) was performed on both  
69 root and soil samples.

70

71 In order to target fungi, we used fungal primers ITS3\_KYO2 (5'-ACACTGACGA CATGGTTCT  
72 ACAGATGAAGAAC GYAGYRAA-3') and ITS4\_KYO3 (5'-TACGGT AGCAGAGACTT GGTCTCTBTTV  
73 CCKCTTCACTCG-3') to produce a final amplicon size of ~430bp. This primer pair should target  
74 the Internal transcribed spacer and inhibit the amplification of plant sequences and enable the  
75 selective amplification of fungal communities from soil, mycorrhizal and other environmental  
76 samples (Toju *et al.* 2012).

77

78 Bacterial primers 341F (5'-CCTACGGG NGGCWGCAG-3') and 805R (5'-GACTACC AGGGTATC  
79 TAATC-3') producing a final amplicon size of ~464b and targeting specifically the bacterial V3-V4  
80 region of the 16S ribosomal gene were chosen given that they has been used extensively in high-  
81 throughput sequencing studies in a range of environments Toju et al. (2012). This primer pair was  
82 shown to be the least biased among 512 primer pairs evaluated in silico for bacterial amplification  
83 Klindworth *et al.* (2013).

84

85 DNA samples were then barcoded, pooled and sequenced (2X300bp, paired-end) using an Illu-  
86 mina MiSeq (San Diego, CA, USA) sequencer at the Genome Quebec Innovation Centre (Montreal,  
87 Canada). Sequences were demultiplexed by the sequencing facility (Genome Quebec Innovation  
88 Centre) and further processed as described below.

89

90

91 *Bioinformatics*

92 All bioinformatics, statistical, and graphic analyses further described were performed in R 3.4.4  
93 (R Core Team 2018) and detailed scripts are available here ([https://github.com/seb951/Acadian\\_SeaPlants](https://github.com/seb951/Acadian_SeaPlants)).  
94

95

96 We used the R package dada2 Callahan et al. (2016) to infer *Amplicon Sequence Variants* (ASVs).  
97 Dada2 offers accurate sample inference from amplicon data with single-nucleotide resolution in  
98 an open source (R) environments. Unlike the Operational Taxonomic Unit (OTU) approach (e.g.  
99 Schloss et al. (2009), Caporaso et al. (2010)), ASV are not treated as cluster of sequences defined  
100 with an *ad hoc* sequence similarity threshold, thus allowing sequences and abundance counts to  
101 be compared among studies Callahan et al. (2016).

102

103 First, sequences were trimmed following strict quality thresholds (see parameter details in the  
104 accompanying R scripts). Following this, we applied the error model algorithm of dada2 which  
105 incorporates quality information after filtering, unlike other OTU based methods. Then derepli-  
106 cation, sample inference, merging of paired end reads and removal of chimera reads were per-  
107 formed in order to obtain a sequence (ASVs) table of abundance per sample. Taxonomy was  
108 also assigned using the Ribosomal Database Project (RDP) Naive Bayesian Classifier algorithm  
109 from Wang et al. (2007). Depending on support (minimum bootstrap support of 80), we as-  
110 signed taxonomy from Kingdom to species. We used the silva database formatted for dada2 to  
111 infer bacterial taxa Callahan (2018) We used the UNITE2017 fasta release (including singletons) to  
112 infer fungal taxa after formatting it to the dada2 format using a custom R script. The pipeline  
113 was run on a multithreaded (48 CPUs) computer infrastructure provided by Westgrid (<https://www.westgrid.ca/support/systems/cedar>) and Compute Canada ([www.computecanada.ca](http://www.computecanada.ca)).  
114 Note that the pipeline was run separately for fungal-root, fungal-soil, bacteria-soil and bacteria-  
115 root samples given the markedly different type of amplicons, taxa and error models of each  
116 dataset.  
117

118

119 *Statistical analyses - plant productivity*

120 We tested for the effect of species (tomato vs pepper), fertilization and their interaction on six

121 plant productivity measures (fruit number, average fruit weight, shoots fresh weight, roots fresh  
122 weight, shoots dry weight, roots dry weight). We used linear mixed effect models (LMM) in the  
123 R package `nlme` Pinheiro et al. (2017), which are more appropriate than an Analysis of Variance  
124 (ANOVA) given the current block design (blocks and replicates nested within a block were treated  
125 as random variables). All six plant productivity measures were square root transformed in order  
126 to help satisfy the assumption of normality of the residuals in the LMM statistical framework.

127

128

129 *Statistical analyses - microbial and fungal diversity*

130 We analysed separately fungal-root, fungal-soil, bacterial-root and bacterial-soil ASV diversity.  
131 For each of these four datasets, we removed samples that showed poor sequencing output and  
132 contained few ASVs. In order to do this, we summed the abundance of all ASVs for each sam-  
133 ple ( $\sum_{i=1}^n ASV$ ) and eliminated samples that had fewer than the mean sum ( $\overline{\sum_{i=1}^n ASV}$ ) -  $4\sigma$  (four  
134 standard deviations). In addition, we removed ASVs from our dataset that were present in fewer  
135 than 5% of the samples (less than ten individuals in the soil samples, and less than five in the root  
136 samples). This was done to remove very rare ASVs which were unique to a block or replicate, but  
137 not found in the majority of a treatment.

138

139 We then conducted community-based analyses looking at the effect of the fertilization treatment  
140 on the abundance ASV taxa in the tomato and pepper experiments. To reduce the complexity of  
141 the datasets, relative abundance of all taxa were calculated per family using the R package `dplyr`  
142 Wickham et al. (2015). Barplots were drawn using `ggplot2` (Hadley 2016) to vizualize the com-  
143 munities. ASV (*a*)-diversity was calculated for each sample using the inverse Simpson diversity  
144 index in `vegan` Oksanen et al. (2013). The effect of fertilization treatment, species (and planting  
145 for soil communities) were assessed using a linear mixed-effect (LMM) model in the R package  
146 `nlme` Pinheiro et al. (2017), given the unbalanced, replicated block design. Alpha diversity was  
147 log transformed in order to help satisfy the assumption of normality of the residuals of the LMM  
148 statistical framework.

149

150 Using the community matrix data of ASVs abundance, we performed PERmutational Multivariate

151 ANalysis Of VAriance tests (PERMANOVA; Anderson (2001)) to identify relationships between  
152 the communities according to the experimental design. ASV abundance data was Hellinger-  
153 transformed and significance was assessed using 10,000 permutations in vegan Oksanen et al.  
154 (2013). Blocks and replicates nested within blocks were factored as strata (blocks) in the model.

155

156 We also performed constrained ordinations (CCAs) using Hellinger-transformed ASV abundance  
157 data in vegan Oksanen et al. (2013) to visually assess the grouping of samples, ASVs and their  
158 association with productivity variables. Data were analysed separately for fungal-root, fungal-  
159 soil, bacterial-root and bacterial-soil, but also according to species (tomato/pepper), given that  
160 analyses of diversity showed that tomato and pepper were markedly different. This gave a total  
161 of eight CCAs. Data were constrained based on four of the productivity measures (fruit number,  
162 average fruits weight, shoots fresh weight, roots fresh weight). We excluded the shoot & root dry  
163 weights as constraints to simplify the model and given that they were highly correlated with the  
164 fresh weight already included as constraints ( $r^2=0.98$  and 0.76 for shoot dry/fresh weights and  
165 root dry/fresh weights, respectively).

166

167 We then identified the ten ASVs most positively associated with the productivity measures of fruit  
168 number, shoots fresh weight and roots fresh weight from each constrained ordinations for a total  
169 of 40 fungal and 40 bacterial candidates ASVs. We aligned sequences using the Bioconductor R  
170 package deciphér Wright (2016) and build pairwise distances matrices using a JC69 substitution  
171 models of DNA sequence evolution (equal base frequencies, Jukes & Cantor (1969)) in phangorn  
172 Schliep (2010). Phylogenetic trees for bacteria and fungi were plotted using ape Paradis, Claude &  
173 Strimmer (2004). This permitted to identify if similar candidate ASVs were found under different  
174 experimental conditions (soil/root, pepper/tomato), thus reinforcing their role in productivity in-  
175 crease, and decreasing the chance that these are false positive.

176

177 **RESULTS**

178 *productivity*

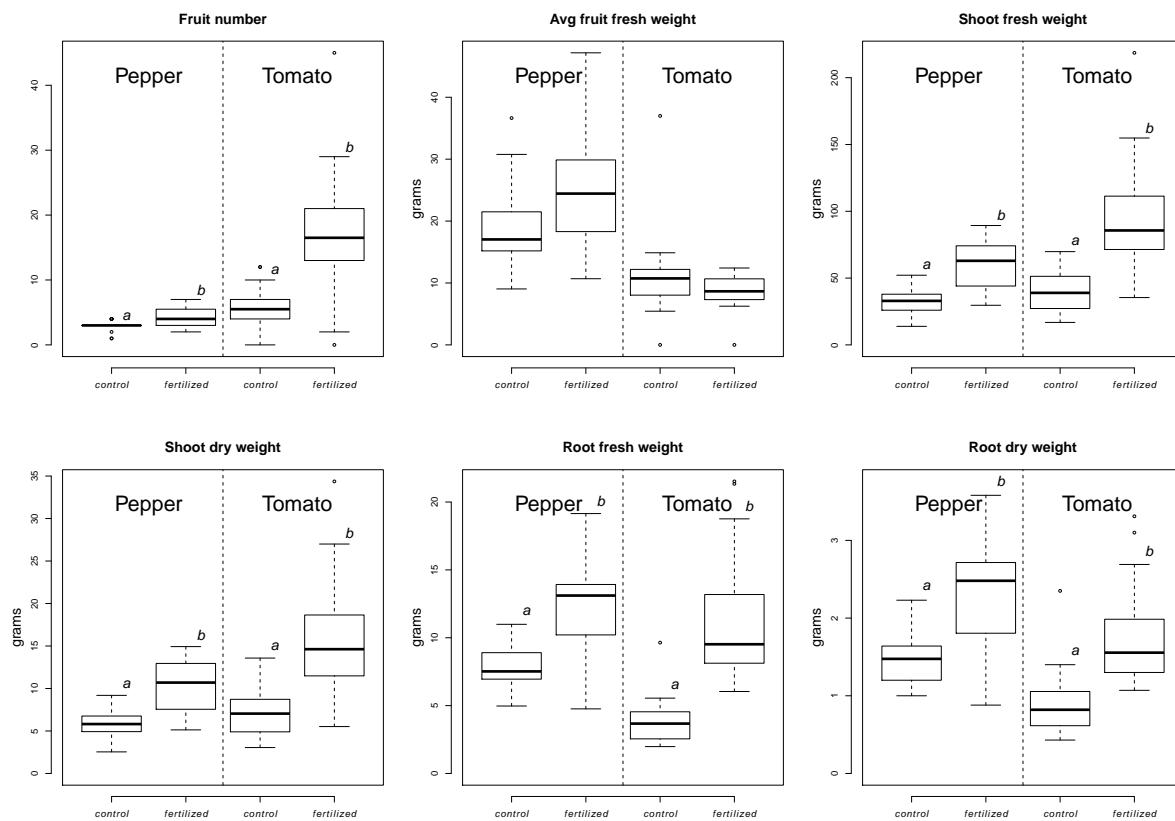
179 We tested the effect of the fertilization treatment on six different measures of overall plant growth  
180 and productivity (fruit number, average fruit weight, shoots fresh weight, roots fresh weight) for  
181 both tomato and peppers. Visually, both above ground and below ground plant structure grew  
182 larger in fertilized plants, in addition to producing more fruits (see Figure 2 for some examples of  
183 the remarkable difference between fertilized and unfertilized plants).



184  
185 **Figure 2: Plant productivity. Photos were taken at the end of the experimental treatment. In**  
186 **each photo, fertilized plants are on the left. A: pepper plants, B: pepper roots, C: pepper fruits**  
187 **and D: tomato fruits.**

188

189 Statistically, all six productivity measures significantly differed according to species, and five of  
190 those were significantly different according to the fertilization treatment. The only exception was  
191 the average fruit weight which did not differ between fertilized and control plants (LMM,  $F_{(1,69)}$   
192 = 1.27,  $p$ -value=0.26). However the model did reveal a significant interaction between treatment  
193 and plant ( $F_{(1,69)} = 9.6$ ,  $p$ -value=0.0028). In fact, when testing only the pepper plants, the effect of  
194 fertilization on average fruit weight was significantly higher in the fertilized pepper plants ( $F_{(1,23)}$   
195 = 10.84,  $p$ -value=0.0032).

198 **Figure 3: measures of plant productivity.**201 *Sequencing*

202 A total of 2.7 million paired-end raw reads were obtained for all samples combined (976,000 for  
 203 fungi-soil, 920,000 for fungi-root, 309,000 for bacteria-soil and 535,000 for bacteria-root, Table 1).  
 204 Note that sequencing samples were analysed separately for fungal-soil, fungal-root, bacteria-soil  
 205 and bacteria-root conditions. On average, 46,965 paired-end reads were obtained per sample,  
 206 and after quality filters were applied, including removing chimeras and paired-end reads were  
 207 merged, an average of 18,435 sequences remained. While 192 soil samples for fungi and bacteria,  
 208 and 92 root samples for fungi and bacteria were sequenced, three fungi-soil samples, 13 fungi-root  
 209 samples and two bacteria-root samples were removed because they had too few reads based on our  
 210 strict quality thresholds.

212 The dada2 pipeline inferred, on average, 112 Amplicon Sequence Variants were inferred per sample  
213 (average of 163 fungal-soil ASV, 49 fungal-root ASVs, 112 bacterial-soil ASVs and 122 bacterial-  
214 root ASVs). Many of those were unique to one of a few samples (total number of 6,178 fungal-soil,  
215 930 fungal-root, 10,120 bacterial-soil and 3,143 bacterial-roots ASVs). In fact, after quality filtering  
216 ASVs that were found in fewer than 10% of the samples, we retained 418, 169, 206 and 250 ASVs  
217 and which comprised 91%, 88%, 50% and 85% of all reads in the fungal soil, fungal roots, bacterial  
218 soil and bacterial roots samples, respectively.

219

220

Table 1: Sequencing and ASV summary

	fungi_soil	fungi_root	bacteria_soil	bacteria_root
Nb_seq_sum	976,000	309,000	920,000	535,000
Nb_seq_mean	51,381	32,208	47,907	56,365
Nb_seq_mean_filtered	38,045	14,635	38,287	46,081
Nb_seq_mean_filt_merged	32,014	13,335	13,780	41,058
Nb_seq_mean_filt_merg_non_chimeras	24,737	8,505	12,049	28,451
Nb_samples	192	96	192	96
Nb_samples_trimmed	189	83	192	94
ASV_sum	6,178	930	10,120	3,143
ASV_sum_trimmed	418	169	206	250
ASV_persample	163	49	112	122

221

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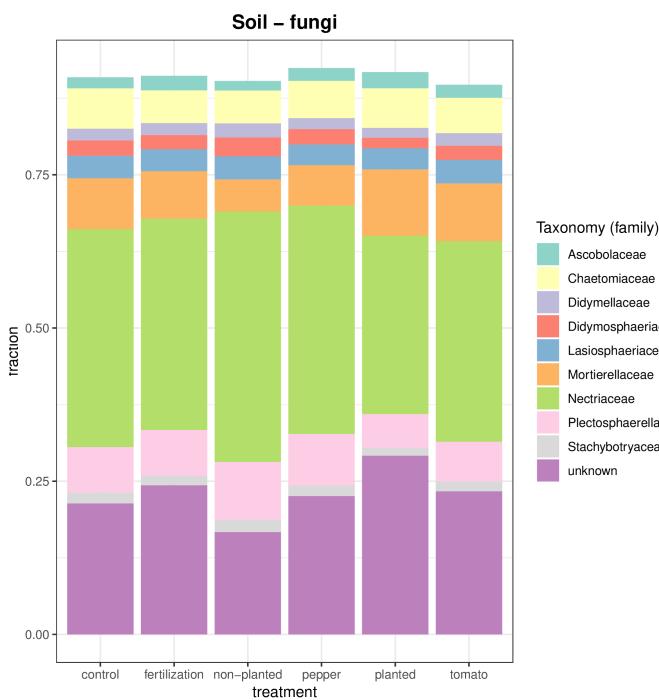
223 *Root & soil microbial and bacterial diversity*

224 We then analysed the whole community structure and report the relative abundance of taxa (fam-  
225 ily) for the fungal-soil, fungal-root, bacteria-soil and bacteria-root conditions (Figure 4). Fungal  
226 communities were dominated by Nectriaceae, both in the root and soil samples. Bacterial

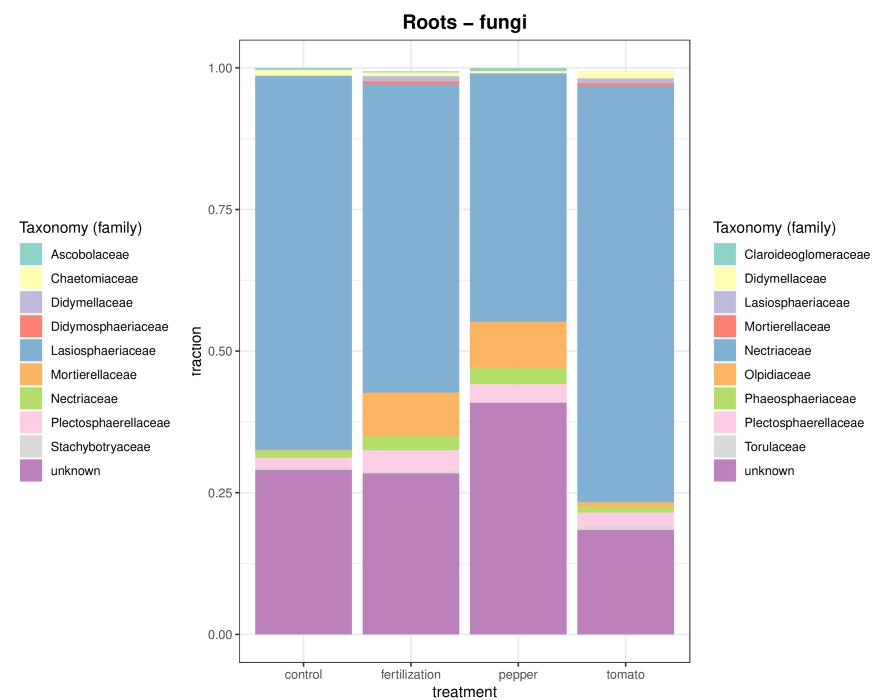
<sup>227</sup> root communities were largely dominated by the Cyanobacteria phylum (identified as *chloroplast*  
<sup>228</sup> according to the Ribosomal Database Project Naive Bayesian Classifier and the silva database).  
<sup>229</sup> In fact, these ASVs are likely chloroplasts from the plant itself that were sequenced, despite the  
<sup>230</sup> fact that the primer pair used should have primarily targeted the bacterial V3-V4 region of the 16S  
<sup>231</sup> ribosomal gene. The bacterial family Bacilaceae dominated to a lesser extent the soil communities.

<sup>232</sup>

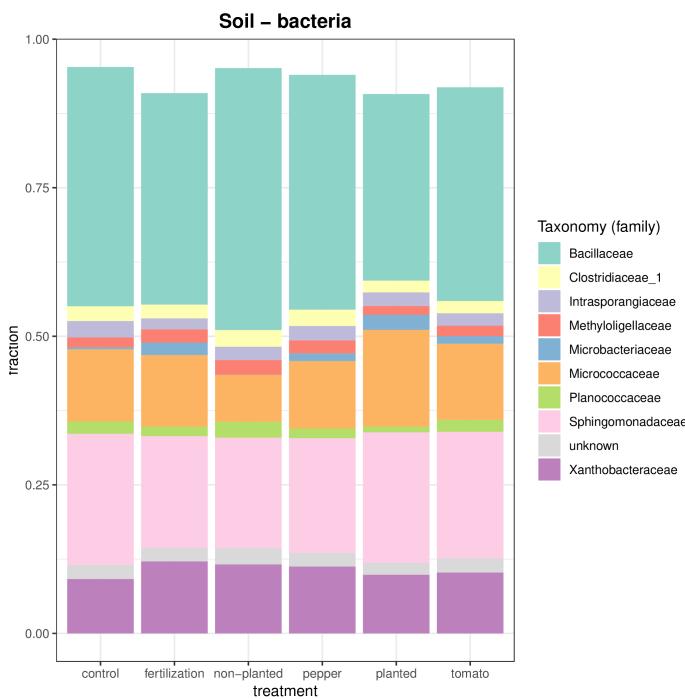
A



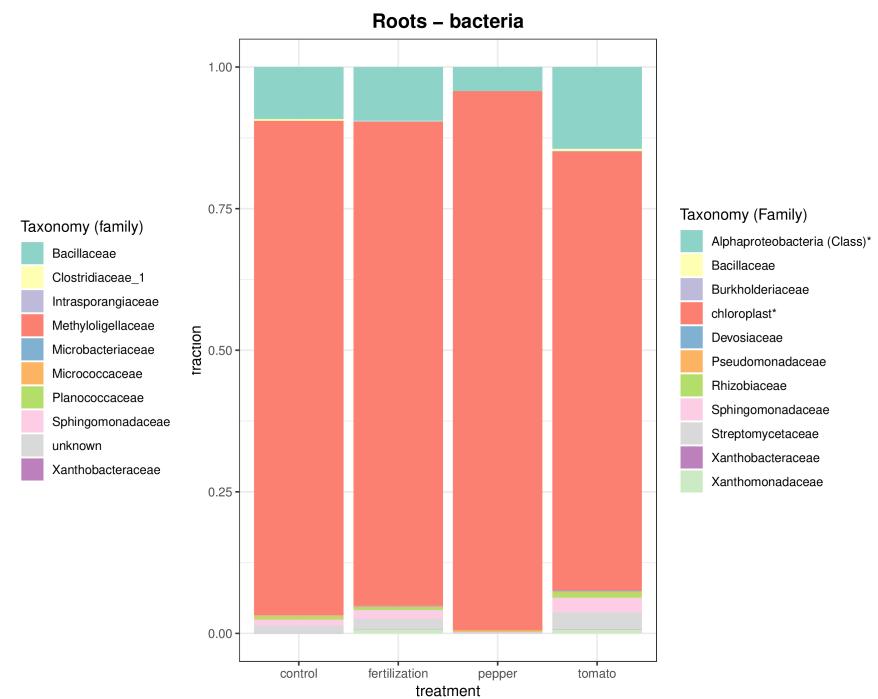
B



C



D



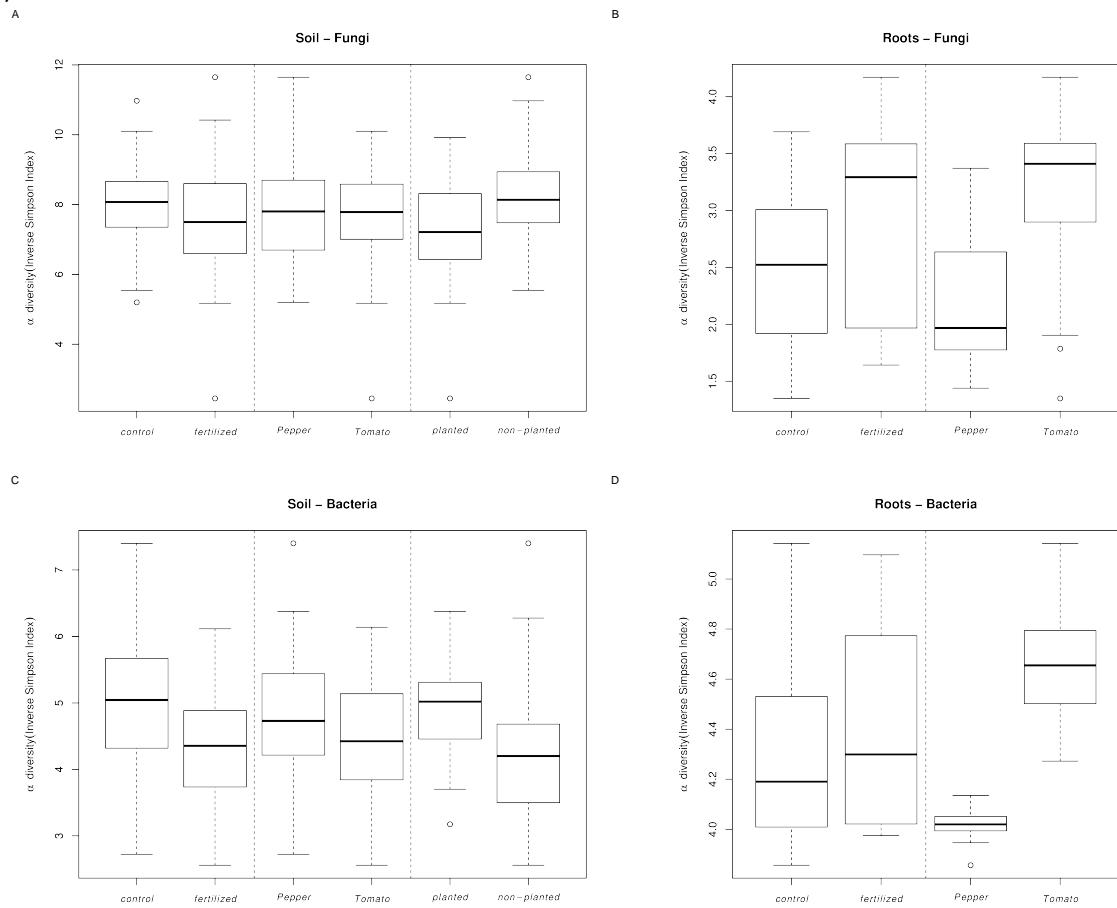
233

234 **Figure 4: Barplots.**

235

237 *Local ( $\alpha$ -diversity)*

238 The diversity of each site ( $\alpha$ -diversity) was calculated separately for each sample and under each  
 239 experimental conditions (fungi-soil, fungi-root, bacteria-soil and bacteria-root, Figure 5). Linear  
 240 mixed effects models used to assess significance. In soils samples, fungal diversity differed with  
 241 respect to the fertilization ( $F_{(1,161)}=14.35, p\text{-value}<0.0001$ ) and planting ( $F_{(1,161)}=41.00, p\text{-value}<0.0001$ )  
 242 treatment, but not the species ( $F_{(1,161)}=0.13, p\text{-value}=0.72$ ). In root samples, fungal diversity dif-  
 243 fered with respect to the fertilization treatment ( $F_{(1,56)}=13.56, p\text{-value}=0.001$ ), and the species tested  
 244 ( $F_{(1,56)}=74.31, p\text{-value}=0.003$ ). In soil samples, bacterial diversity differed with respect to the fer-  
 245 tilization treatment ( $F_{(1,165)}=46.25, p\text{-value}<0.0001$ ), planting ( $F_{(1,165)}=48.77, p\text{-value}<0.0001$ ) and  
 246 species ( $F_{(1,165)}=10.22, p\text{-value}=0.002$ ). In root samples, bacterial diversity differed with respect  
 247 to the fertilization treatment ( $F_{(1,67)}=16.48, p\text{-value}=0.0001$ ), and the species tested ( $F_{(1,67)}=523.42,$   
 248  $p\text{-value}<0.0001$ ).



250 **Figure 5: Barplots.**

251

252

253 *Differences in species composition among sites*

254 Using a PERMANOVA statistical framework, we identified that for all conditions, communities  
255 differed with respect to the fertilization treatment (Table 2). Soil fungal and bacterial communities  
256 differed the most according to whether the tray was planted (greatest % of variance explained,  
257 Table 2), while root communities differed the most between tomato and pepper plants.

Table 2: summary of PERMANOVAs\*

	fungi_soil	fungi_root	bacteria_soil	bacteria-root
fertilization	0.02 (4e-04)	0.04 (0.0013)	0.03 (1e-04)	0.01 (0.0705)
planted	0.15 (1e-04)	NA	0.06 (1e-04)	NA
species	0.02 (2e-04)	0.2 (1e-04)	0.01 (0.0032)	0.42 (1e-04)
fertilization:planted	0.01 (0.008)	NA	0.02 (1e-04)	NA
fertilization:species	0.01 (0.0705)	0.03 (0.0094)	0.01 (0.002)	0.01 (0.0973)
planted:species	0.01 (0.1597)	NA	0.01 (0.1767)	NA
fertilization:planted:species	0 (0.7956)	NA	0.01 (0.1179)	NA

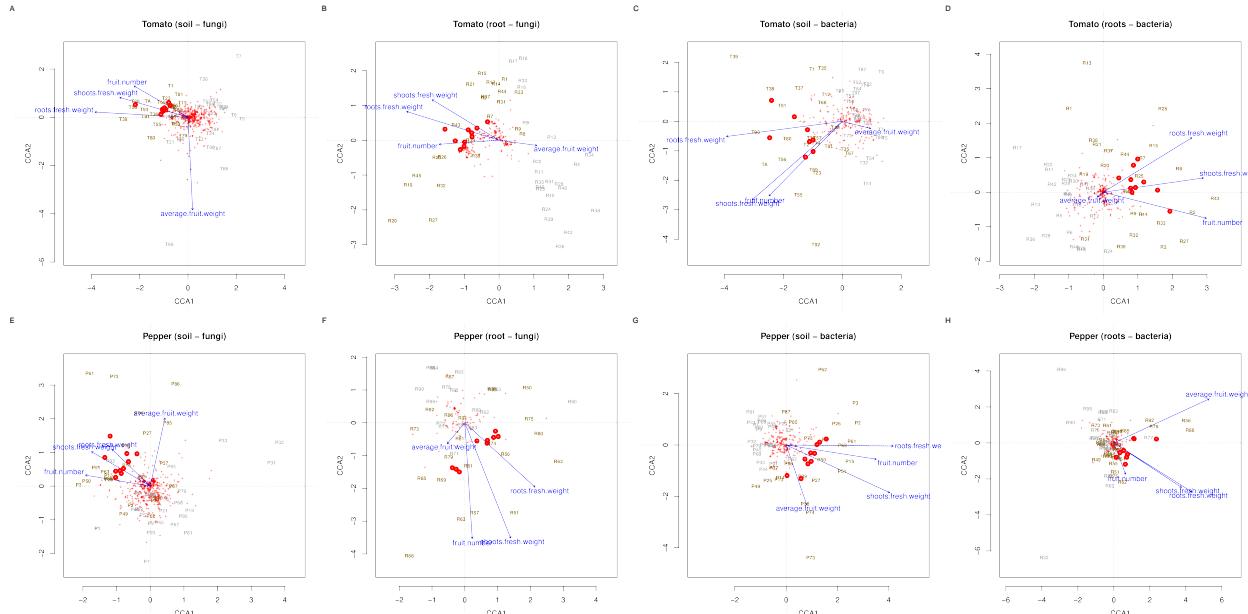
258 \* $R^2$  [percentage of variance explained by the term in the model] and associated p-values in parentheses

259

260 *Constrained ordinations and candidate ASVs*

261 Constrained ordinations clearly indicated how fertilized samples clustered together according to  
262 their fungal or bacterial communities (Figure 6). It also shows how three of the constrain variables  
263 (productivity measures of root fresh weight, shoots fresh weight and fruit number) were asso-  
264 ciated with the fertilization treatment, while average fruit weight behave differentially (in fact  
265 nearly orthogonally to the other three constrains in most ordinations).

266



267

268 **Figure 6: Constrained ordinations.** Samples are labelled and colored in gray (unfertilized) or  
 269 dark yellow (fertilized). Red crosses represent individual ASVs, while red points represent the  
 270 ten ASVs most closely associated with the three productivity measures of fruit number, shoots  
 271 fresh weight and root freshweight. Blue arrows are the four productivity measures used as  
 272 constraints in the ordinations.

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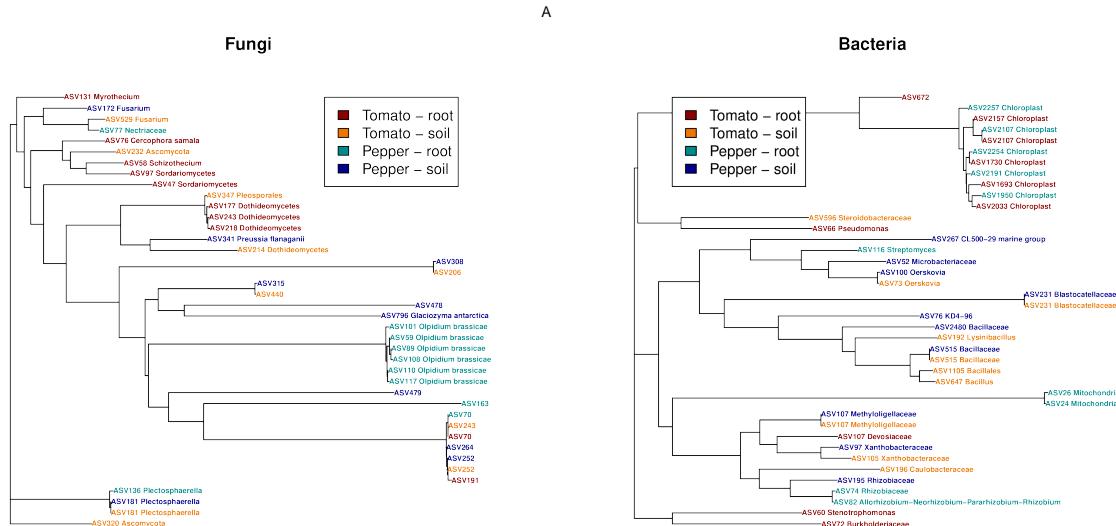
275 Next, we identified, for each ordination, the ten ASVs most closely related to the three constraints  
 276 which behaved in a similar fashion (productivity measures of root fresh weight, shoots fresh  
 277 weight and fruit number). These were considered as putative candidates most positively impacted  
 278 (increase presence of the ASV) by fertilization. We further analysed the corresponding sequences  
 279 for these eighty candidates (ten candidates \* eight ordinations) ASVs in two separate alignments  
 280 (one for fungi and one for bacterial ASVs) and neighbouring joining trees. In fungi, we identified  
 281 one cluster of ASVs taxonomically assigned to *Olpidium brassicae* (fungal obligate parasite in the  
 282 phylum Chytridiomycota) that forms the majority of ASVs most closely related to productivity. In  
 283 addition, we identified five different ASVs in both species and both root and soil, closely related  
 284 phylogenetically. Given that no taxonomy was assigned to these sequences through the dada2  
 285 RDP bootstrap approach, we used a BLASTn approach (against NCBI nr) to identify the most

286 closely related sequences. We identified this cluster of ASVs as *Rhogostoma schuessleri* (BLASTn,  
287 e-value=2e-74), a protist in the phylum Cercozoa, which are known to be present in the soil and  
288 phyllosphere Dumack et al. (2017)

289

In bacteria-roots, we identified a cluster of ten closely related sequences taxonomically assigned to *Chloroplast*, and which likely originate from the plant themselves. We also identified a number of ASVs associated with productivity in the soil of both the pepper and tomato plants. Notably, ASV100 & ASV73 (*Oerskovia* spp.), ASV231 (*Blastocatellaceae*), ASV515, ASV1105 & ASV647 (*Bacillaceae*), ASV107 (*Methyloligellaceae*) and ASV95 & ASV107 (*Santhobacteraceae*) were identified.

295



296

297 **Figure 7: Neighbor-Joining trees of candidates ASVs (fungi & roots) associated with produc-  
298 tivity measures**

299

300

<sup>301</sup> **DISCUSSION**

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