

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

4 *true*

5 *September 12, 2018*

6 **Abstract**

7 The abstract will be written here

8 INTRODUCTION

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

13
14 The aim of this project was to develop a better understanding of the effects of *A. nodosum* extracts
15 on plant growth. We tested the effect of these extract on two commonly used plants (Tomato -
16 *Solanum lycopersicum* and Pepper - *Capsicum annuum*) using different measures of productivity. In
17 addition, we tested how the bacterial and fungal communities responded to the addition of *A.
18 nodosum* extracts.

19

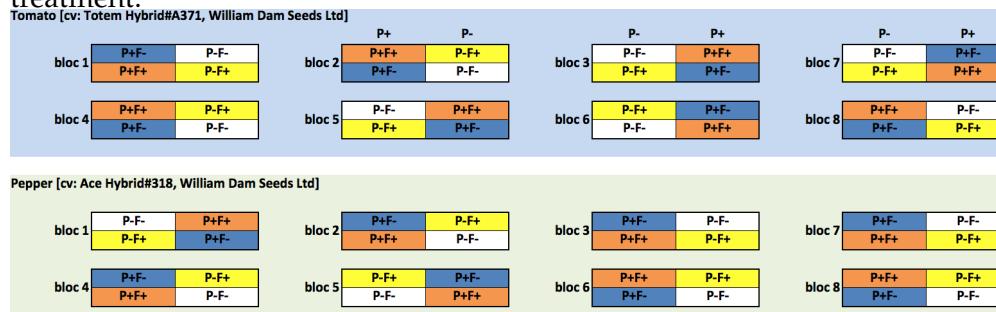
20 MATERIAL AND METHOD

21 Study design

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

28

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



35

36 Figure 1: experimental design

37

38 Tomato plants were fertilized using multipurpose organic fertilizer (pure hen manure, 18 g per tray
39 repeated every 4 weeks, 5-3-2) from Acti-sol (Notre-Dame-du-Bon-Conseil, Qc, Canada) in addition
40 to Stella Maris® (3.5 ml per 1L, each tray received 250 ml, repeated every 2 weeks) for the duration
41 of the experiment. Stella Maris® is a registered trademark from Acadian Seaplants Ltd. (Darmouth,
42 NS, Canada). It is primarily composed of *Ascophyllum nodosum* seaweed and is advertized as

43 a natural activator of the crops' own growth and defense mechanisms to improve root growth
44 and resist temperature, drought, and salinity stress in order to maximize yield and crop qualities
45 (Acadian Seaplants Ltd. 2018). Pepper plants were fertilized using solely Stella Maris (3.5 ml per
46 1L, each tray received 250 ml, repeated every 2 weeks) for the duration of the experiment.

47
48 Thrips were managed with *Neoseiulus cucumeris* (syn. *Amblyseius cucumeris*) (100 bags), Fungus
49 gnat and thrips were also controlled using predatory mite *Gaeolaelaps gillespiei* (1L). Plants were
50 treated once a week with Oidium Milstop.

51

52

53 *Plant productivity*

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

60

61

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

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

68

69 In order to target fungi, we used fungal primers ITS3_KYO2 (5'-ACACTGACGA CATG-
70 GTTCT ACAGATGAAGAAC GYAGYRAA-3') and ITS4_KYO3 (5'-TACGGT AGCAGAGACTT
71 GGTCTCTBTTV CCKCTTCACTCG-3') to produce a final amplicon size of ~430bp. This primer
72 pair should target the Internal transcribed spacer and inhibit the amplification of plant sequences
73 and enable the selective amplification of fungal communities from soil, mycorrhizal and other
74 environmental samples (Toju *et al.* 2012).

75

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

82

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

87

88

89 *Bioinformatics*

90 All bioinformatics, statistical, and graphic analyses further described were performed in R 3.4.4 (R

91 Core Team 2018) and detailed scripts are available here (https://github.com/seb951/Acadian_Seaplants).
92

93
94 We used the R package dada2 Callahan et al. (2016) to infer *Amplicon Sequence Variants (ASVs)*.
95 Dada2 offers accurate sample inference from amplicon data with single-nucleotide resolution in
96 an open source (R) environments. Unlike the Operational Taxonomic Unit (OTU) approach (e.g.
97 Schloss et al. (2009), Caporaso et al. (2010)), ASV are not treated as cluster of sequences defined
98 with an *ad hoc* sequence similarity threshold, thus allowing sequences and abundance counts to be
99 compared among studies Callahan et al. (2016).

100
101 First, sequences were trimmed following strict quality thresholds (see parameter details in
102 the accompanying R scripts). Following this, we applied the error model algorithm of dada2
103 which incorporates quality information after filtering, unlike other OTU based methods. Then
104 dereplication, sample inference, merging of paired end reads and removal of chimera reads
105 were performed in order to obtain a sequence (ASVs) table of abundance per sample. Taxonomy
106 was also assigned using the Ribosomal Database Project (RDP) Naive Bayesian Classifier
107 algorithm from Wang et al. (2007). Depending on support (minimum bootstrap support of
108 80), we assigned taxonomy from Kingdom to species. We used the silva database formatted
109 for dada2 to infer bacterial taxa Callahan (2018) We used the Community (2018) fasta release
110 (including singletons) to infer fungal taxa after formatting it to the dada2 format using a
111 custom R script. The pipeline was run on a multithreaded (48 CPUs) computer infrastructure
112 provided by Westgrid (<https://www.westgrid.ca/support/systems/cedar>) and Compute Canada
113 (www.computecanada.ca). Note that the pipeline was run separately for fungal-root, fungal-soil,
114 bacteria-soil and bacteria-root samples given the markedly different type of amplicons, taxa and
115 error models of each dataset.

116
117 *Statistical analyses - plant productivity*
118 We tested for the effect of species (tomato vs pepper), fertilization and their interaction on six plant
119 productivity measures (fruit number, average fruit weight, shoots fresh weight, roots fresh weight,
120 shoots dry weight, roots dry weight). We used linear mixed effect models (LMM) in the R package
121 nlme Pinheiro et al. (2017), which are more appropriate than an Analysis of Variance (ANOVA)
122 given the current block design (blocks and replicates nested within a block were treated as random
123 variables). All six plant productivity measures were square root transformed in order to help
124 satisfy the assumption of normality of the residuals in the LMM statistical framework.

125
126
127 *Statistical analyses - microbial and fungal diversity*
128 We analysed separately fungal-root, fungal-soil, bacterial-root and bacterial-soil ASV diversity.
129 For each of these four datasets, we removed samples that showed poor sequencing output and
130 contained few ASVs. In order to do this, we summed the abundance of all ASVs for each sample
131 ($\sum_{i=1}^n \text{ASV}$) and eliminated samples that had fewer than the mean sum ($\overline{\sum_{i=1}^n \text{ASV}}$) - 4σ (four
132 standard deviations). In addition, we removed ASVs from our dataset that were present in fewer
133 than 5% of the samples (less than ten individuals in the soil samples, and less than five in the root
134 samples). This was done to remove very rare ASVs which were unique to a block or replicate, but
135 not found in the majority of a treatment.

136
137 We then conducted community-based analyses looking at the effect of the fertilization treatment

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

147
148 Using the community matrix data of ASVs abundance, we performed PERmutational Multivariate
149 ANalysis Of VAriance tests (PERMANOVA; Anderson (2001)) to identify relationships between
150 the communities according to the experimental design. ASV abundance data was Hellinger-
151 transformed and significance was assessed using 10,000 permutations in `vegan` Oksanen et al.
152 (2013). Blocks and replicates nested within blocks were factored as strata (blocks) in the model.

153
154 We also performed constrained ordinations (CCAs) using Hellinger-transformed ASV abundance
155 data in `vegan` Oksanen et al. (2013) to visually assess the grouping of samples, ASVs and their
156 association with productivity variables. Data were analysed separately for fungal-root, fungal-soil,
157 bacterial-root and bacterial-soil, but also according to species (tomato/pepper), given that analyses
158 of diversity showed that tomato and pepper were markedly different. This gave a total of eight
159 CCAs. Data were constrained based on four of the productivity measures (fruit number, average
160 fruits weight, shoots fresh weight, roots fresh weight). We excluded the shoot & root dry weights
161 as constraints to simplify the model and given that they were highly correlated with the fresh
162 weight already included as constraints ($r^2=0.98$ and 0.76 for shoot dry/fresh weights and root
163 dry/fresh weights, respectively).

164
165 We then identified the ten ASVs most positively associated with the productivity measures of fruit
166 number, shoots fresh weight and roots fresh weight from each constrained ordinations for a total
167 of 40 fungal and 40 bacterial candidates ASVs. We aligned sequences using the Bioconductor R
168 package `decipher` Wright (2016) and build pairwise distances matrices using a JC69 substitution
169 models of DNA sequence evolution (equal base frequencies, Jukes & Cantor (1969)) in `phangorn`
170 Schliep (2010). Phylogenetic trees for bacteria and fungi were plotted using `ape` Paradis, Claude &
171 Strimmer (2004). This permitted to identify if similar candidate ASVs were found under different
172 experimental conditions (soil/root, pepper/tomato), thus reinforcing their role in productivity
173 increase, and decreasing the chance that these are false positive.

174

175 **RESULTS**

176 *productivity*

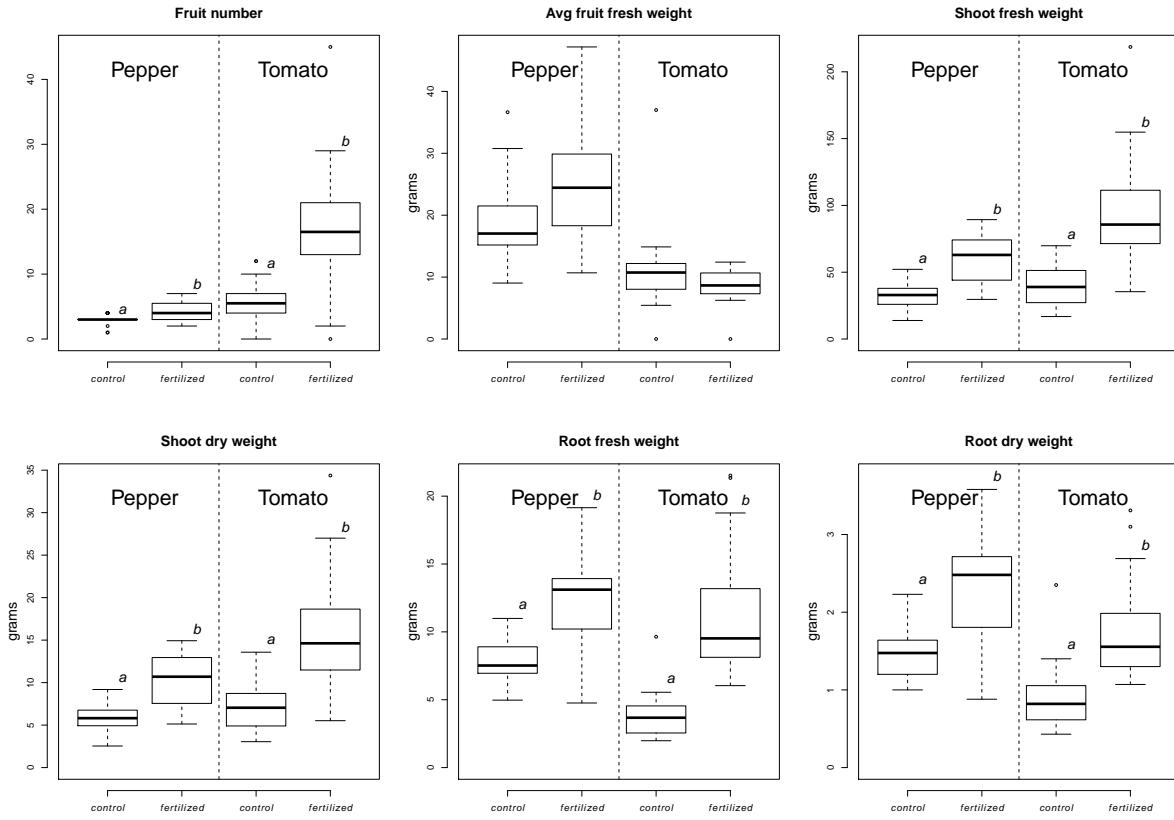
177 We tested the effect of the fertilization treatment on six different measures of overall plant growth
178 and productivity (fruit number, average fruit weight, shoots fresh weight, roots fresh weight) for
179 both tomato and peppers. Visually, both above ground and below ground plant structure grew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



182
183 **Figure 2: Plant productivity.** Photos were taken at the end of the experimental treatment. In
184 each photo, fertilized plants are on the left. A: pepper plants, B: pepper roots, C: pepper fruits
185 and D: tomato fruits.

186
187 Statistically, all six productivity measures significantly differed according to species, and five of
188 those were significantly different according to the fertilization treatment. The only exception was
189 the average fruit weight which did not differ between fertilized and control plants (LMM, $F_{(1,69)}$
190 = 1.27, p -value=0.26). However the model did reveal a significant interaction between treatment
191 and plant ($F_{(1,69)} = 9.6$, p -value=0.0028). In fact, when testing only the pepper plants, the effect of
192 fertilization on average fruit weight was significantly higher in the fertilized pepper plants ($F_{(1,23)}$
193 = 10.84, p -value=0.0032).

194



195
196 **Figure 3: measures of plant productivity.**
197

198 *Sequencing*

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

208
209 The dada2 pipeline inferred, on average, 112 Amplicon Sequence Variants were inferred per sample
210 (average of 163 fungal-soil ASV, 49 fungal-root ASVs, 112 bacterial-soil ASVs and 122 bacterial-root
211 ASVs). Many of those were unique to one of a few samples (total number of 6,178 fungal-soil, 930
212 fungal-root, 10,120 bacterial-soil and 3,143 bacterial-roots ASVs). In fact, after quality filtering ASVs
213 that were found in fewer than 10% of the samples, we retained 418, 169, 206 and 250 ASVs and
214 which comprised 91%, 88%, 50% and 85% of all reads in the fungal soil, fungal roots, bacterial soil
215 and bacterial roots samples, respectively.

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

219

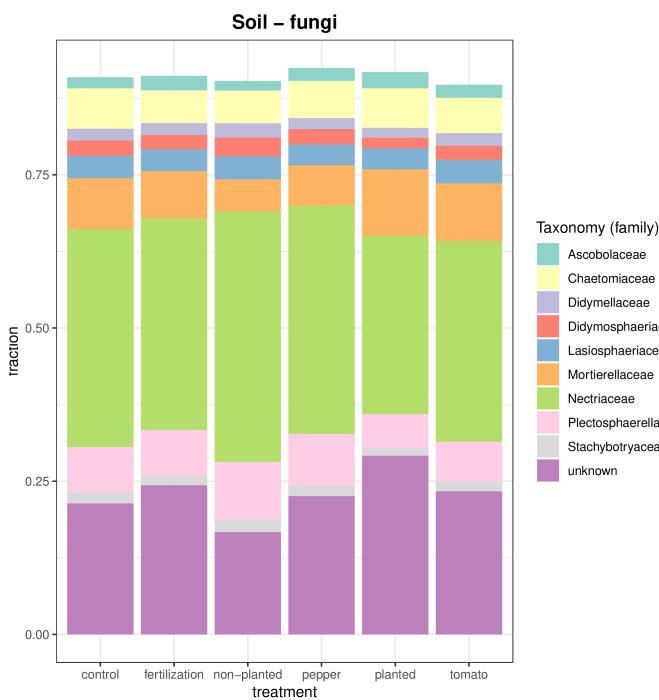
220

221 *Root & soil microbial and bacterial diversity*

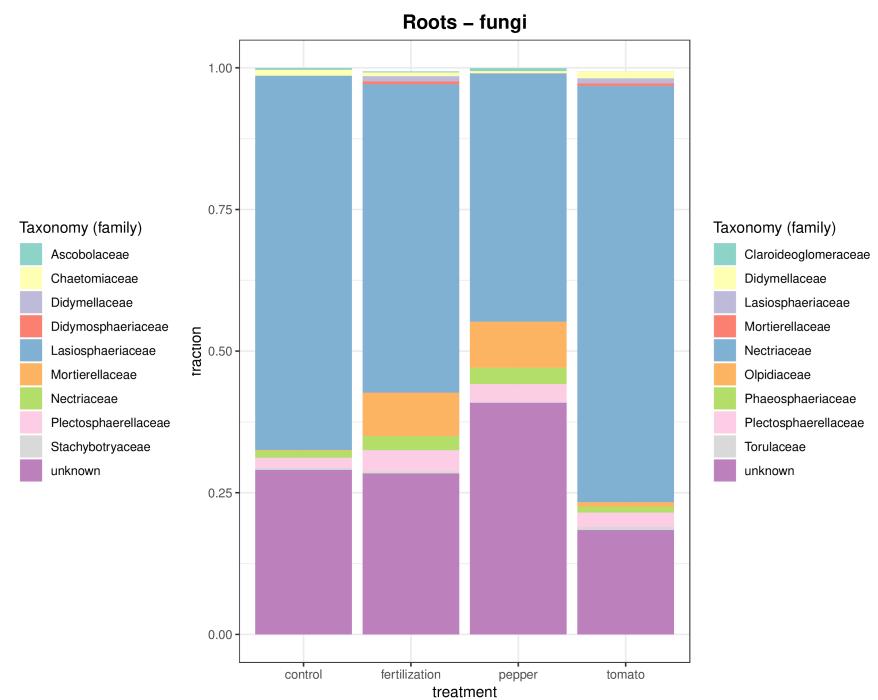
222 We then analysed the whole community structure and report the relative abundance of taxa
 223 (family) for the fungal-soil, fungal-root, bacteria-soil and bacteria-root conditions (Figure 4). Fungal
 224 communities were dominated but Nectriaceae, both the in the root and soil samples. Bacterial
 225 root communities were largely dominated by the Cyanobacteria phylum (identified as *chloroplast*
 226 according to the Ribosomal Database Project Naive Bayesian Classifier and the silva database).
 227 In fact, these ASVs are likely chloroplasts from the plant itself that were sequenced, despite the
 228 fact that the primer pair used should have primarily targeted the bacterial V3-V4 region of the 16S
 229 ribosomal gene. The bacterial family Bacillaceae dominated to a lesser extent the soil communities.

230

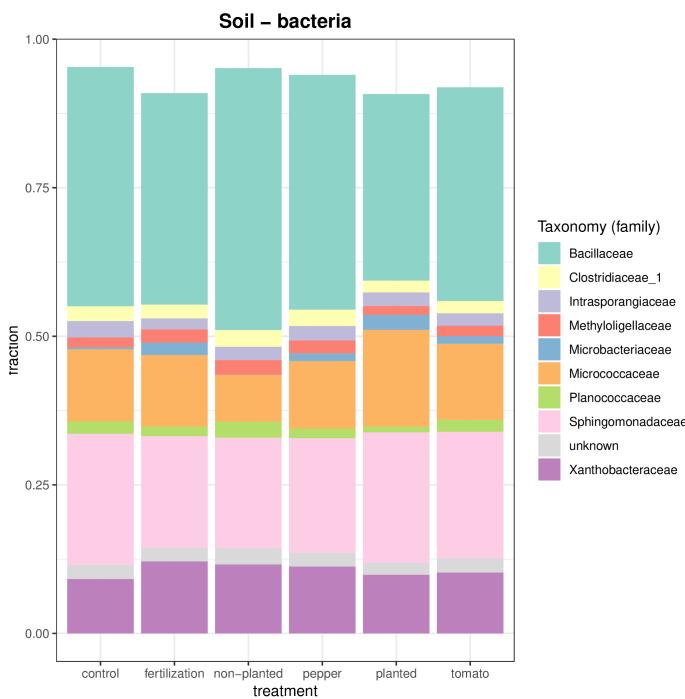
A



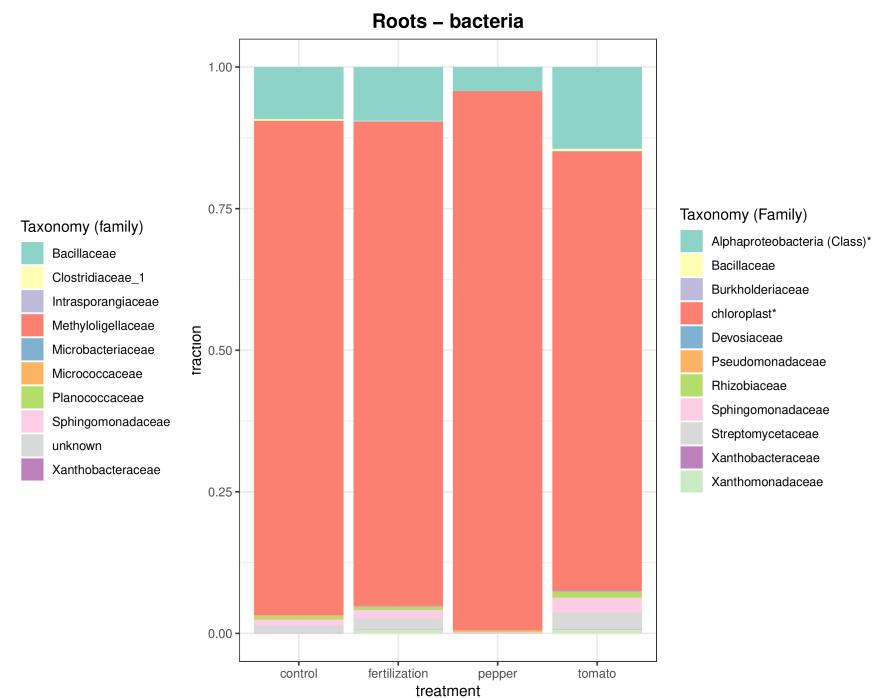
B



C



D



231

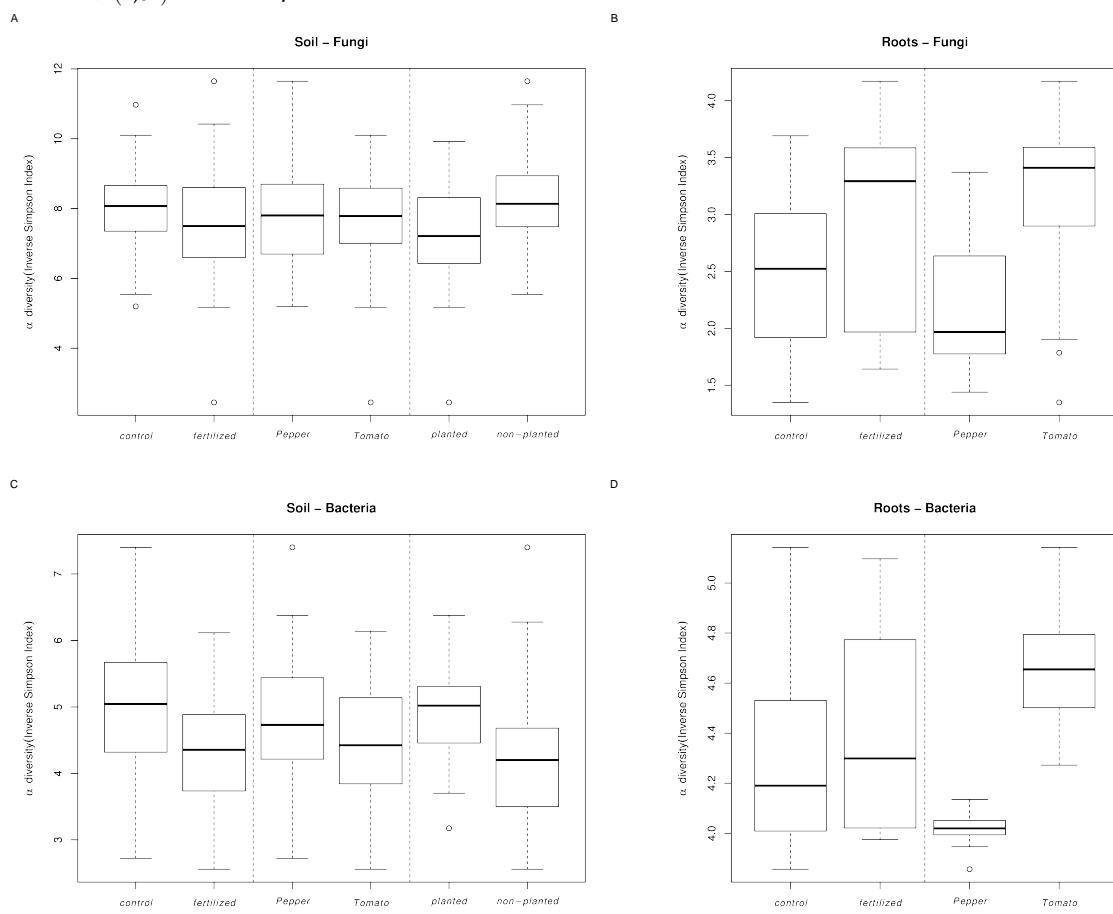
Figure 4: Barplots.

233

234

235 Local (α -diversity)

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



247

248 **Figure 5: Barplots.**

249

250

251 *Differences in species composition among sites*

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

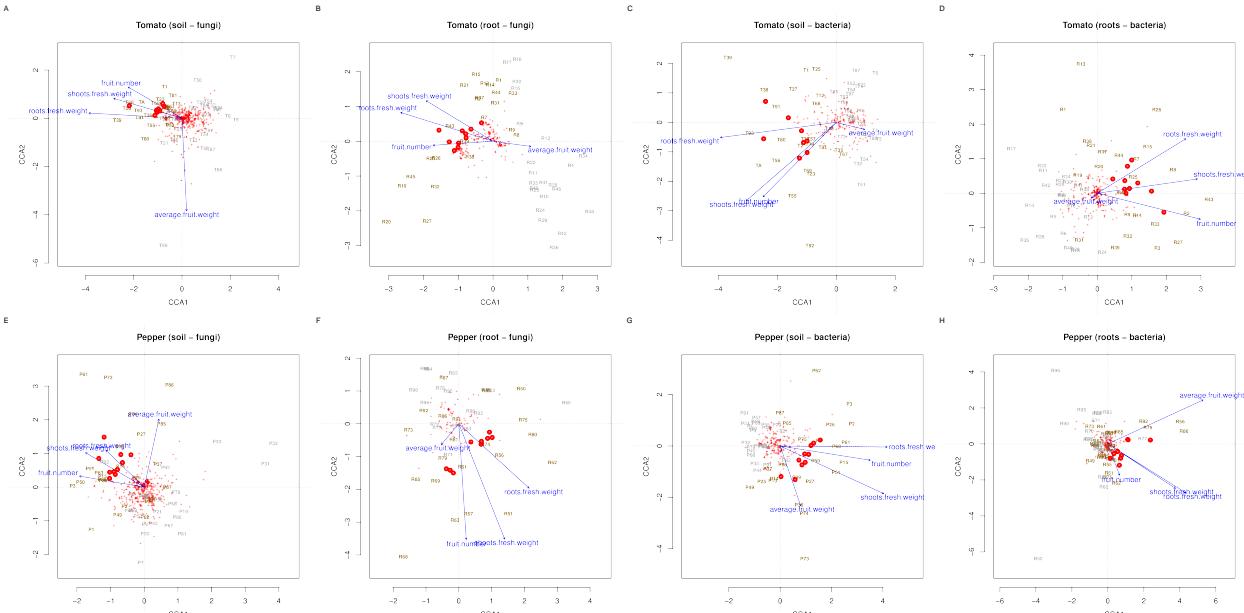
* R^2 [percentage of variance explained by the term in the model] and associated p-values in parentheses

257

258 Constrained ordinations and candidate ASVs

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

264



265

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

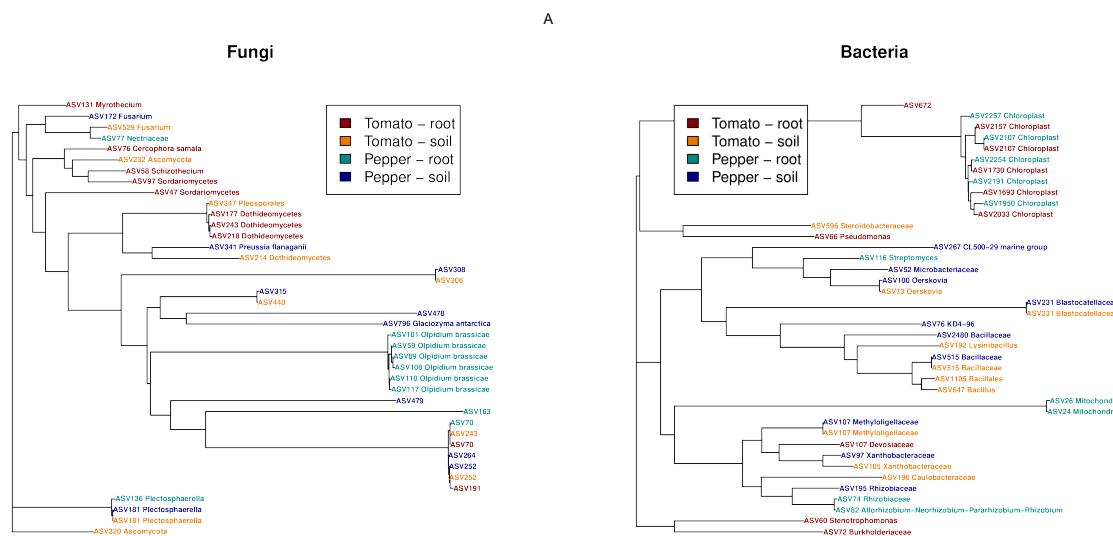
271

272

273 Next, we identified, for each ordination, the ten ASVs most closely related to the three constraints
274 which behaved in a similar fashion (productivity measures of root fresh weight, shoots fresh

275 weight and fruit number). These were considered as putative candidates most positively impacted
276 (increase presence of the ASV) by fertilization. We further analysed the corresponding sequences
277 for these eighty candidates (ten candidates * eight ordinations) ASVs in two separate alignments
278 (one for fungi and one for bacterial ASVs) and neighbouring joining trees. In fungi, we identified
279 one cluster of ASVs taxonomically assigned to *Olpidium brassicae* (fungal obligate parasite in the
280 phylum Chytridiomycota) that forms the majority of ASVs most closely related to productivity. In
281 addition, we identified five different ASVs in both species and both root and soil, closely related
282 phylogenetically. Given that no taxonomy was assigned to these sequences through the dada2
283 RDP bootstrap approach, we used a BLASTn approach (against NCBI nr) to identify the most
284 closely related sequences. We identified this cluster of ASVs as *Rhogostoma schuessleri* (BLASTn,
285 e-value=2e-74), a protist in the phylum Cercozoa, which are known to be present in the soil and
286 phyllosphere Dumack et al. (2017)

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.



294
295 **Figure 7: Neighbor-Joining trees of candidates ASVs (fungi & roots) associated with produc-
296 tivity measures**

²⁹⁸ (look at *m vpart* package? *m vpartwrapt MRT()* function)

²⁹⁹ **DISCUSSION**

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