

<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* algae 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

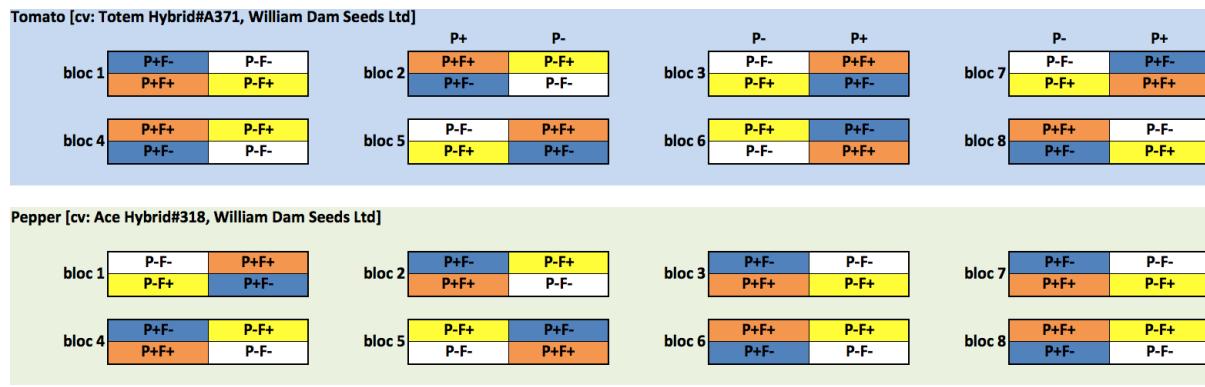
**21 MATERIAL AND METHOD**

**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 from an agricultural field under organic regime  
26 at the IRDA research station in St-Bruno (Qc, Canada) on October 7<sup>th</sup> 2015 (loamy sand soil, 15 cm  
27 top layer collected). Soil characteristics (pH, conductivity, nutrients, see Supplementary Table 1)  
28 were measured by AgriDirect (Longueuil, Qc, Canada).

29

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



36

37 **Figure 1: experimental design**

38

39 Half of the tomato plants were fertilized using multipurpose organic fertilizer (pure hen ma-  
 40 nure, 18 g per tray repeated every 4 weeks, 5-3-2) from Acti-sol (Notre-Dame-du-Bon-Conseil,  
 41 Qc, Canada) in addition to Stella Maris® (3.5 ml per 1L, each tray received 250 ml, repeated every  
 42 2 weeks) for the duration of the experiment. The other half were unfertilized. Stella Maris® is a  
 43 registered trademark from Acadian Seaplants Ltd. (Darmouth, NS, Canada). It is primarily com-  
 44 posed of *Ascophyllum nodosum* seaweed and is advertised as a natural activator of the crops' own  
 45 growth and defense mechanisms to improve root growth and resist temperature, drought, and  
 46 salinity stress in order to maximize yield and crop qualities (Acadian Seaplants Ltd. 2018). Half  
 47 of the pepper plants were treated using solely Stella Maris (3.5 ml per 1L, each tray received 250  
 48 ml, repeated every 2 weeks) for the duration of the experiment. The other half were untreated.  
 49 Thrips were managed with *Neoseiulus cucumeris* (syn. *Amblyseius cucumeris*) (100 bags), Fungus  
 50 gnat and thrips were also controlled using predatory mite *Gaeolaelaps gillespiei* (1L). Plants were  
 51 treated once a week with Oidium Milstop to control the fungus.

52

53

54 *Plant productivity*

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

60 expected to represent well the plant overall productivity.

61

62

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

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

69

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

76

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

83

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

88

89

90 *Bioinformatics*

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

94

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

101

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

117

118 *Statistical analyses - plant productivity*

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

120 productivity measures (fruit number, average fruit weight, shoots fresh weight, roots fresh weight,  
121 shoots dry weight, roots dry weight). We used linear mixed effect models (LMM) in the R package  
122 `nlme` Pinheiro et al. (2017), which are more appropriate than an Analysis of Variance (ANOVA)  
123 given the current block design (blocks and replicates nested within a block were treated as random  
124 variables). All six plant productivity measures were either square root or log transformed in or-  
125 der to help satisfy the assumption of normality of the residuals in the LMM statistical framework.  
126 For the variables *fruit number* and *average fruit weight*, we also used a permutation-based 2-way  
127 ANOVA (Anderson & Legendre (1999)) given that the residuals of the LMM were not normally  
128 distributed, and results were similar.

129

130

### 131 *Statistical analyses - microbial and fungal diversity*

132 We analysed separately fungal-root, fungal-soil, bacterial-root and bacterial-soil ASV diversity.  
133 For each of these four datasets, we removed samples that showed poor sequencing output and  
134 contained few ASVs. In order to do this, we summed the abundance of all ASVs for each sam-  
135 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  
136 standard deviations). In addition, we removed ASVs from our dataset that were present in fewer  
137 than 5% of the samples (less than ten individuals in the soil samples, and less than five in the root  
138 samples). This was done to remove very rare ASVs which were unique to a block or replicate, but  
139 not found in the majority of a treatment.

140

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

150 statistical framework.

151

152 Using the community matrix data of ASVs abundance, we performed PERmutational Multivariate  
153 ANalysis Of VAriance tests (PERMANOVA; Anderson (2001)) to identify relationships between  
154 the communities according to the experimental design. ASV abundance data was Hellinger-  
155 transformed and significance was assessed using 10,000 permutations in vegan Oksanen et al.  
156 (2013). Blocks and replicates nested within blocks were factored as strata (blocks) in the model.

157

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

168

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

179

180 **RESULTS**

181 *productivity*

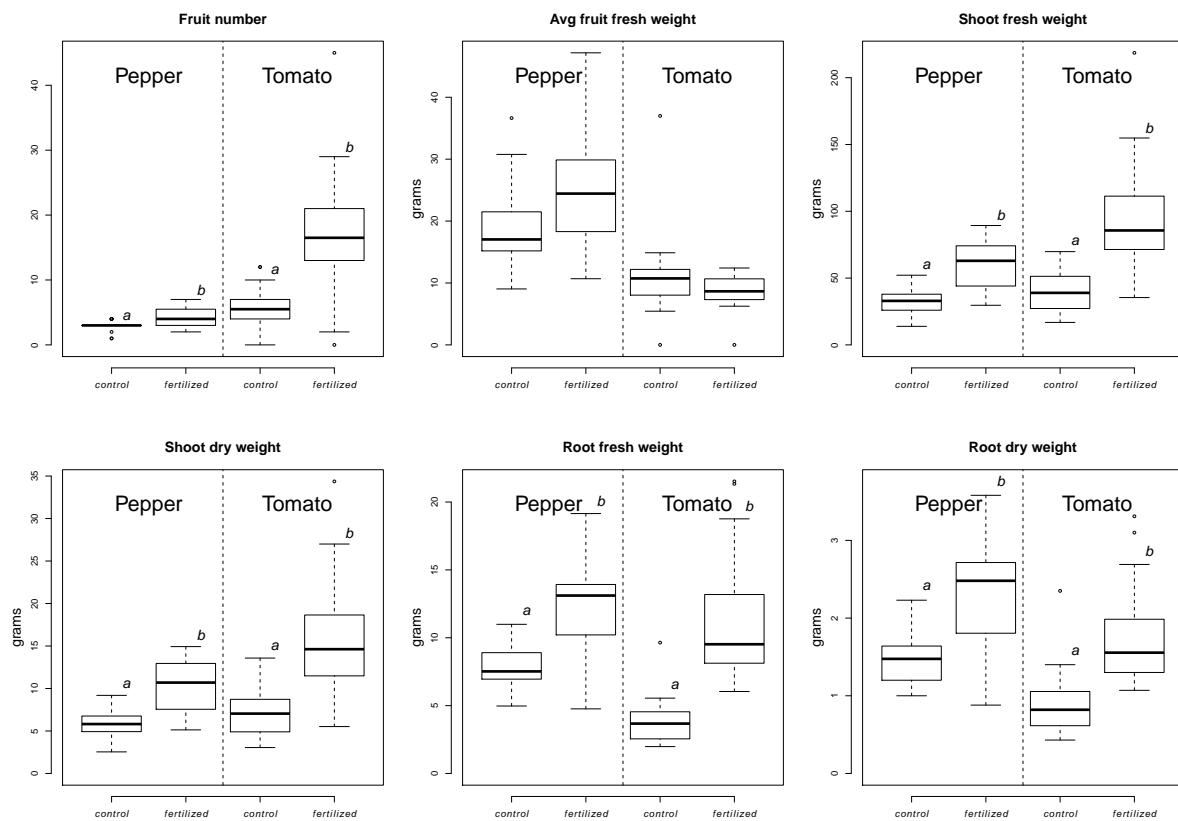
182 We tested the effect of the fertilization treatment on six measures of overall plant growth and pro-  
183 ductivity (fruit number, average fruit weight, shoots fresh weight, shoots dry weight, roots fresh  
184 weight, roots dry weight) for both tomato and peppers. Visually, both above ground and below  
185 ground plant structure grew larger in fertilized plants, in addition to producing more fruits (see  
186 Figure 2 for some examples of the striking difference between fertilized and unfertilized plants).



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

191

192 Statistically, all six productivity measures significantly differed according to species, and five of  
193 those were significantly different according to the fertilization treatment. The only exception was  
194 the average fruit weight which did not differ between fertilized and control plants (LMM,  $F_{(1,69)}$   
195 = 1.27,  $p$ -value=0.26). However the model did reveal a significant interaction between treatment  
196 and plant ( $F_{(1,69)} = 9.6$ ,  $p$ -value=0.0028). In fact, when testing only the pepper plants, the effect of  
197 fertilization on average fruit weight was significantly higher in the fertilized pepper plants ( $F_{(1,23)}$   
198 = 10.84,  $p$ -value=0.0032).

201 **Figure 3: measures of plant productivity.**204 *Sequencing*

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

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

222

223

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

224

225

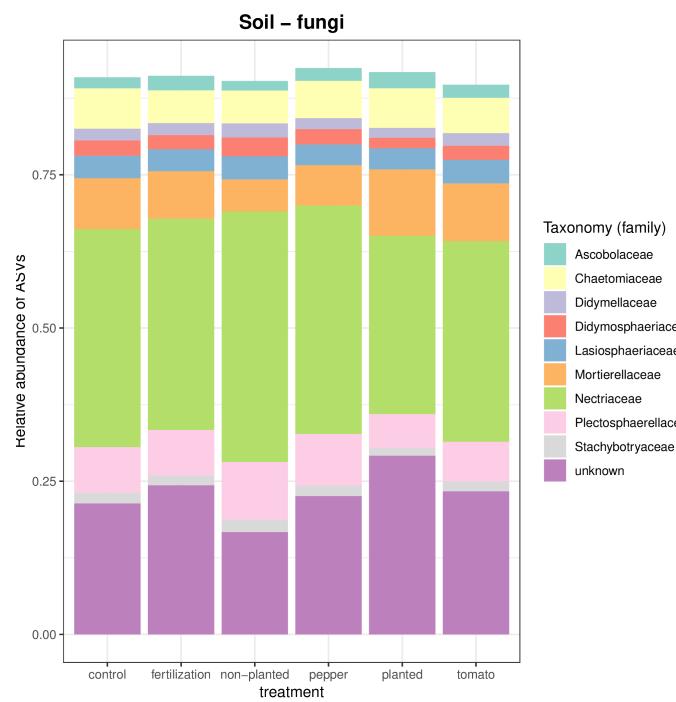
226 *Root & soil microbial and bacterial diversity*

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

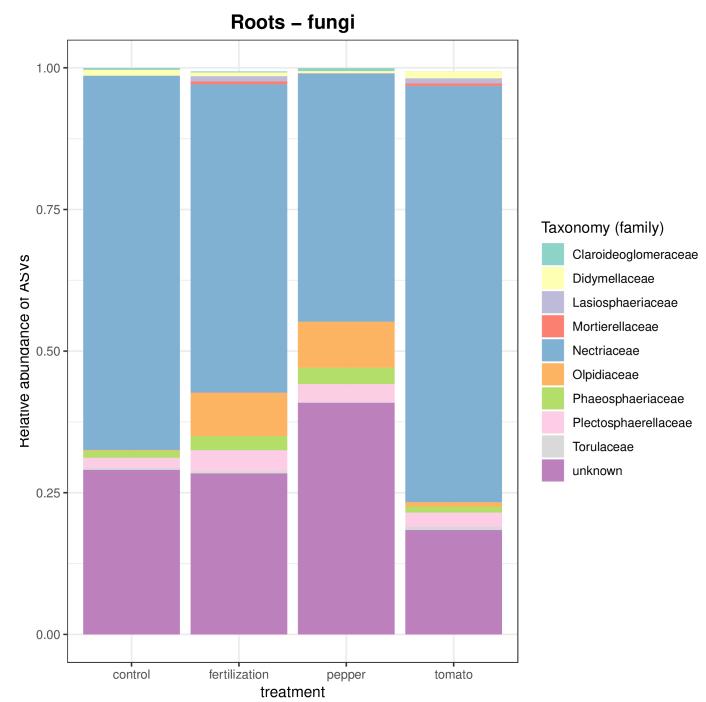
230 communities were largely dominated by the Cyanobacteria phylum (identified as *chloroplast* ac-  
231 cording to the Ribosomal Database Project Naive Bayesian Classifier and the silva database). In  
232 fact, these ASVs are likely sequenced chloroplasts from the plants themselves, despite the fact that  
233 the primer pair used should have primarily targeted the bacterial V3-V4 region of the 16S riboso-  
234 mal gene. The bacterial family Bacilaceae dominated to a lesser extent the soil communities.

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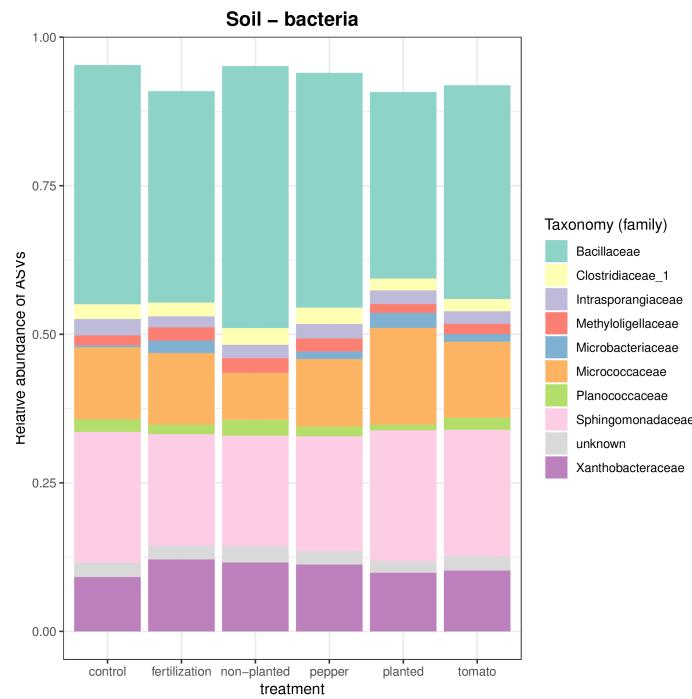
A



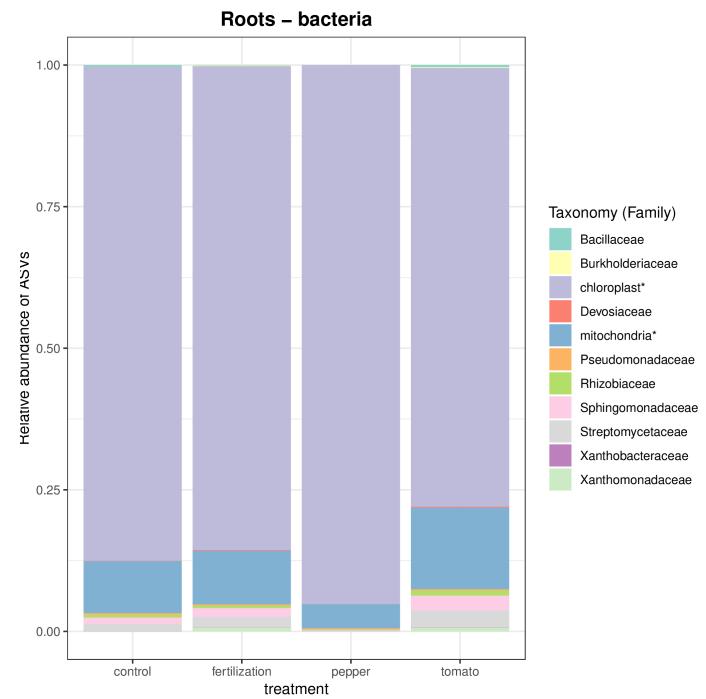
B



C



D



236

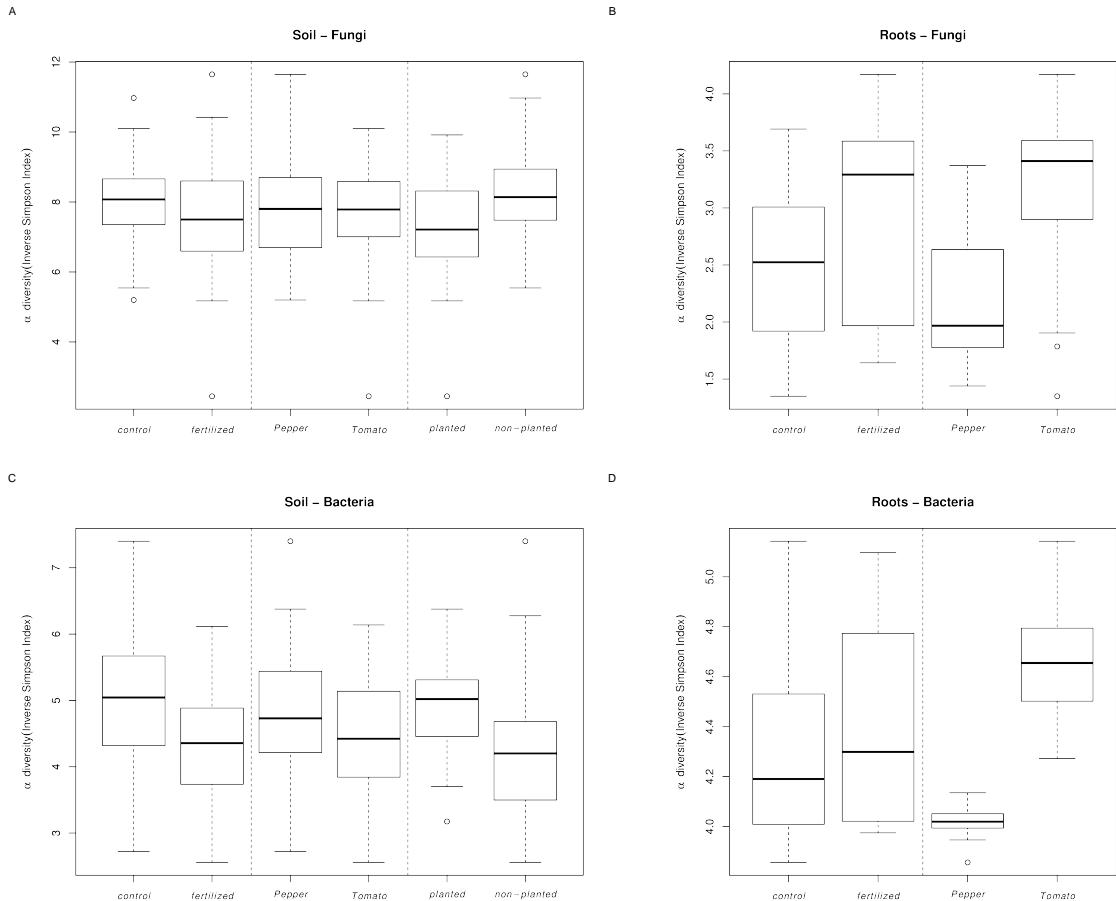
237 **Figure 4: Barplots fo the relative abundance of ASVs for fungal-root, fungal-soil, bacteria-soil**238 **and bacteria-root**

239

240

241 *Local (a-diversity)*

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



253

254 **Figure 5: Boxplot of alpha diversity according to the treatment, species and planting effect for**  
 255 **fungal-root, fungal-soil, bacteria-soil and bacteria-root.**

256

257

258 *Differences in species composition among sites*

259 Using a PERMANOVA statistical framework, we identified that for all conditions, communities  
 260 differed with respect to the fertilization treatment (Table 2). Soil fungal and bacterial communities  
 261 differed the most according to whether the tray was planted (greatest % of variance explained,  
 262 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)

	fungi-soil	fungi-root	bacteria-soil	bacteria-root
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

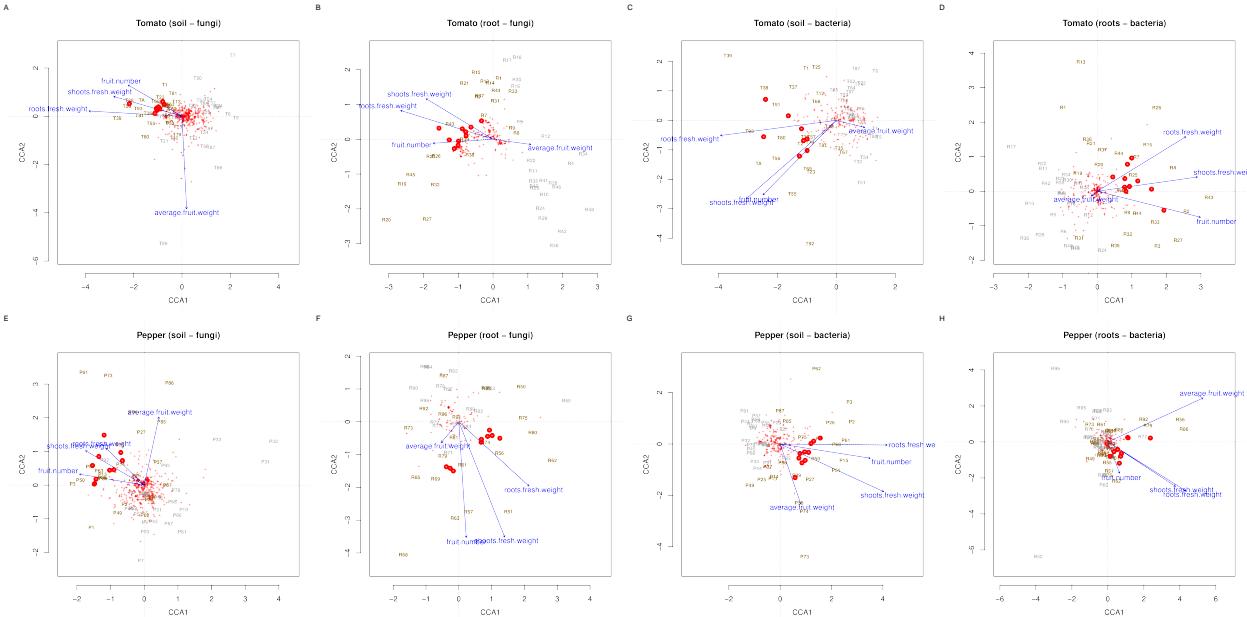
263     \* $r^2$  [percentage of variance explained by the term in the model] and associated  $p$ -values in  
 264     parentheses.

265

#### 266     *Constrained ordinations and candidate ASVs*

267     Constrained ordinations indicated how fertilized samples clustered together according to their  
 268     fungal or bacterial communities (Figure 6). It also shows a similar association of three of the con-  
 269     strain variables (productivity measures of root fresh weight, shoots fresh weight and fruit num-  
 270     ber), while average fruit weight behave differentially (in fact nearly orthogonally to the other three  
 271     constraints in most ordinations).

272



273

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

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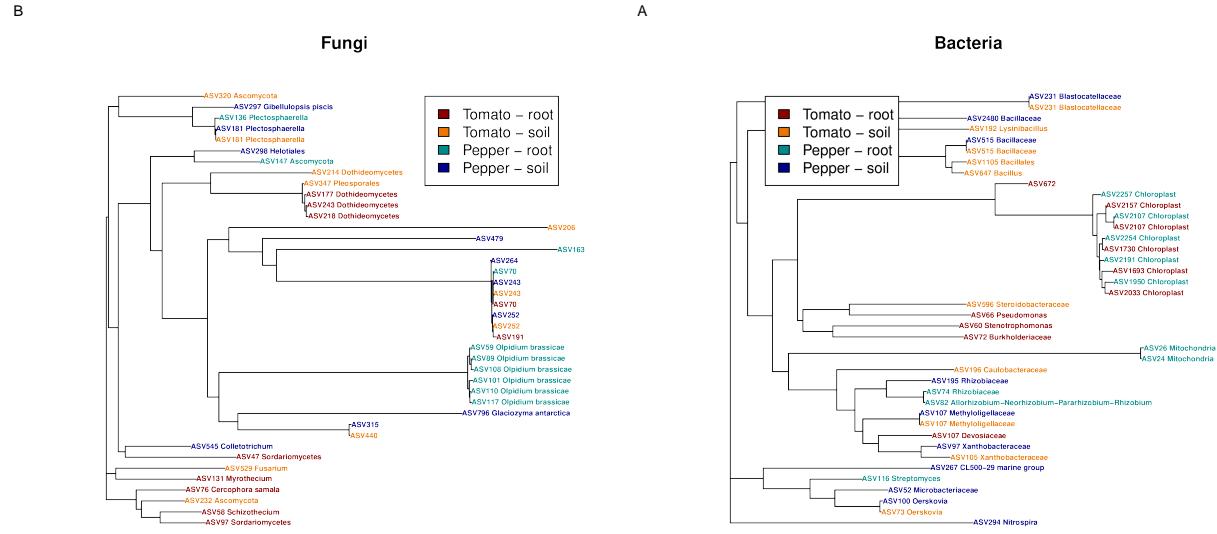
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281 Next, we identified, for each ordination, the ten ASVs most closely related to the three con-  
282 strains which behaved in a similar fashion (productivity measures of root fresh weight, shoots  
283 fresh weight and fruit number). These ASVs were considered as putative candidates sequences  
284 most positively impacted (increase presence of the ASV) by fertilization. We further analysed the  
285 corresponding sequences for these eighty candidates (ten candidates \* eight ordinations) ASVs  
286 in two separate alignments (one for fungi and one for bacterial ASVs) and their accompanying  
287 neighbouring joining trees. In fungi, we identified one cluster of ASVs taxonomically assigned to  
288 *Olpidium brassicae* (fungal obligate parasite in the phylum Chytridiomycota) that forms the ma-  
289 jority of ASVs most closely related to productivity. In addition, we identified five different ASVs  
290 in both species and both root and soil closely related phylogenetically. Given that no taxonomy  
291 was assigned to these sequences through the dada2 RDP bootstrap approach, we used a BLASTn  
292 Altschul et al. (1997) approach (against NCBI nr) to identify the most closely related sequences.  
293 We identified this cluster of ASVs as *Rhogostoma schuessleri* (BLASTn, *e-value*=2e-74), a protist in  
294 the phylum Cercozoa, which are known to be present in the soil and phyllosphere Dumack et al.  
295 (2017).

296

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

302



303

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

306

307 **DISCUSSION**

- 308 • *Brief recap of soil characteristics*
- 309 • *Overall increases in productivity in both species (but mention that tomato were fertilized with hen manure*
- 310 *as well).* • *Talk about effect of treatment on root + soil diversity.*
- 311 • *Talk about the fact in the roots, we most likely sequenced the plant itself, rather than the bacteria.*
- 312 • *Discuss some of the candidate ASVs from Figure 7.*

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