

¹ **The effect of *Ascophyllum nodosum* extracts on plant productivity and fungal and bacterial communities.**

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⁶ The abstract will be written here

⁷ *Keywords:* Stella Marris, 16S, ITS, microbial diversity, Illumina MiSeq

8 INTRODUCTION

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

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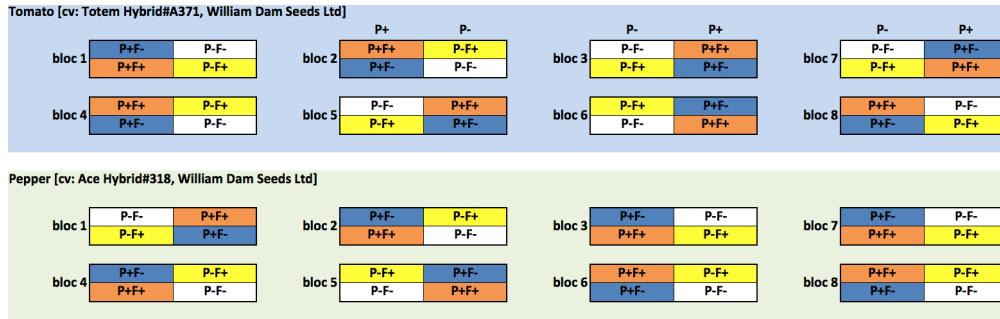
20 MATERIAL AND METHOD

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* and Pepper - *Capsicum annuum*), a ran-
30 domized split block design (Figure 1) was used with four trays set up per block (eight blocks).
31 Half of 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, 18g per
 39 tray repeated every 4 weeks, 5-3-2) from Acti-sol (Notre-Dame-du-Bon-Conseil, Qc, Canada) in
 40 addition to Stella Maris® (3.5 ml per 1L, each tray received 250 ml, repeated every 2 weeks) for
 41 the duration of the experiment. Stella Maris® is a registered trademark from Acadian Seaplants
 42 Ltd. (Darmouth, NS, Canada). It is primarily composed of *Ascophyllum nodosum* seaweed and is
 43 advertised as a natural activator of the crops' own growth and defense mechanisms to improve
 44 root growth and resist temperature, drought, and salinity stress in order to maximize yield and
 45 crop qualities (ref.). Pepper plants were fertilized using solely Stella Maris (3.5 ml per 1L, each
 46 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 cho-
 56 sen randomly per tray (for each treatment [fertilization/control], species [tomato/pepper] and
 57 block [eight blocks]) for a total of 96 samples. In addition, both shoots and roots were dried in a
 58 70 degrees drying oven, and dry weights were measured after 48 hours. Together, these traits are
 59 expected to represent well the plant overall productivity (ref.).

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
64 was extracted using XXX DNA isolation kit with Yg of soil. Roots were first washed with sterile
65 water and DNA was extracted using XXX DNA isolation kit with Yg 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'-ACACTGACGACATGGTTCT
70 ACAGATGAAGAACGYAGYRAA-3') and ITS4_KYO3 (5'-TACGGTAGCAGAGACTT GGTCTCTBTTVCCCKCTT
71 3') to produce a final amplicon size of 430bp (Toju *et al.* 2012).

72

73 Bacterial primers 341F (5'-CCTACGGGNNGCWGCAG-3') and 805R (5'-GACTACCAGGGTATC
74 TAATC-3') producing a final amplicon size of ~464b and targeting specifically the bacterial V3-V4
75 region of the 16S ribosomal gene were chosen given that they has been used extensively in high-
76 throughput sequencing studies in a range of environments (Hugerth *et al.* 2014). This primer pair
77 was shown to be the least biased among 512 primer pairs evaluated in silico for bacterial amplifi-
78 cation (Klindworth *et al.* 2012).

79

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

84

85

86 *Bioinformatics*

87 All bioinformatics, statistical, and graphic analyses further described were performed in R 3.4.4
88 (R Core Team 2018) and detailed scripts are available here (https://github.com/seb951/Acadian_Seaplants).

90

91 We used the R package dada2 (Callahan *et al.* 2015) to infer *Amplicon Sequence Variants* (ASVs).
92 Dada2 offers accurate sample inference from amplicon data with single-nucleotide resolution
93 in an open source (R) environments. Unlike the Operational Taxonomic Unit (OTU) approach
94 (e.g. Schloss *et al.* 2009, Caporaso *et al.* 2010), ASV are not treated as cluster of sequences defined
95 with an *ad hoc* sequence similarity threshold, thus allowing sequences and abundance counts to
96 be compared among studies (Callahan *et al.* 2015).

97

98 First, sequences were trimmed following strict quality thresholds (see parameter details in the
99 accompanying R scripts). Following this, we applied the error model algorithm of dada2 which
100 incorporates quality information after filtering, unlike other OTU based methods. Then derepli-
101 cation, sample inference, merging of paired end reads and removal of chimera reads were per-
102 formed in order to obtain a sequence (ASVs) table of abundance per sample. Taxonomy was
103 also assigned using the Ribosomal Database Project (RDP) Naive Bayesian Classifier algorithm
104 from Wang *et al.* (2007). Depending on support (minimum bootstrap support of 80), we assigned
105 taxonomy from Kingdom to species. We used the silva database formatted for dada2 to infer bac-
106 terial taxa (Callahan 2018). We used the UNITE (2017) fasta release (including singletons) to infer
107 fungal taxa after formatting it to the dada2 format using a custom R script. The dada2 pipeline
108 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).
109 Note that the pipeline was run separately for fungal-root, fungal-soil, bacteria-soil and bacteria-
110 root samples given the markedly different type of amplicons, taxa and error models of each
111 dataset.
112

113

114 *Statistical analyses - plant productivity*

115 We tested for the effect of species (tomato vs pepper), fertilization and their interaction on six
116 plant productivity measures (fruit number, average fruit weight, shoots fresh weight, roots fresh
117 weight, shoots dry weight, roots dry weight). We used linear mixed effect models (LMM) in the R
118 package NLME (Pinheiro *et al.* 2017), which are more appropriate than ANOVAs given the current
119 block design (blocks and replicates nested within a block were treated as random variables). All

¹²⁰ six plant productivity measures were square root transformed in order to help satisfy the assumption
¹²¹ of normality of the residuals in the LMM statistical framework.

¹²²

¹²³

¹²⁴ *Statistical analyses - microbial and fungal diversity*

¹²⁵ We analysed separately fungal-root, fungal-soil, bacterial-root and bacterial-soil ASV diversity.
¹²⁶ For each of these four datasets, we removed samples that showed poor sequencing output and
¹²⁷ contained few ASVs. In order to do this, we summed the abundance of all ASVs for each sample ($\sum_{i=1}^n ASV$) and eliminated samples that had fewer than the mean sum ($\overline{\sum_{i=1}^n ASV}$) - 4σ (four
¹²⁸ standard deviations). In addition, we removed ASVs from our dataset that were present in fewer
¹²⁹ than 5% of the samples (less than 10 individuals in the soil samples, and less than 5 in the root
¹³⁰ samples). This was done to remove very rare ASVs which were unique to a block or replicate, but
¹³¹ not found in the majority of a treatment.

¹³³

¹³⁴ We then conducted community-based analyses looking at the effect of the fertilization treatment
¹³⁵ on the abundance ASV taxa in the tomato and pepper experiments. To reduce the complexity of
¹³⁶ the datasets, relative abundance of all taxa were calculated per family using dplyr (Wickham *et*
¹³⁷ *al.* 2015). Barplots were drawn using ggplot2 (Hadley 2016) to visualize the communities. ASV
¹³⁸ (a)-diversity was calculated for each sample using the inverse Simpson diversity index in the R
¹³⁹ package VEGAN (Oksanen *et al.* 2013). The effect of fertilization treatment, species (and planting
¹⁴⁰ for soil communities) were assessed using a linear mixed-effect (LMM) model in the R package
¹⁴¹ NLME (Pinheiro *et al.* 2017), given the unbalanced, replicated block design. Alpha diversity was
¹⁴² log transformed in order to help satisfy the assumption of normality of the residuals of the LMM
¹⁴³ statistical framework.

¹⁴⁴

¹⁴⁵ Using the community matrix data of ASVs abundance, we performed PERmutational Multivariate
¹⁴⁶ ANalysis Of VAriance tests (PERMANOVA; Anderson 2001) to identify relationships between
¹⁴⁷ the communities according to the experimental design. ASV abundance data was Hellinger-
¹⁴⁸ transformed and significance was assessed using 10,000 permutations in VEGAN (Oksanen *et*
¹⁴⁹ *al.* 2013). Blocks and replicates nested within blocks were factored as strata (blocks) in the model.

150

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

161

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

171

172 [A partition of the variation was also performed to assess how much of the variation was ex-
173 plained by the soil and the vegetation characteristics. I didn't include this in the end...]

174 **RESULTS**

175 *productivity*

176 We tested the effect of the fertilization treatment on six different measures of overall plant growth
177 and productivity (fruit number, average fruit weight, shoots fresh weight, roots fresh weight) for
178 both tomato and peppers. Fertilized plants grew better and this is apparent both visually (Figure
179 2) and statistically (Figure 3).

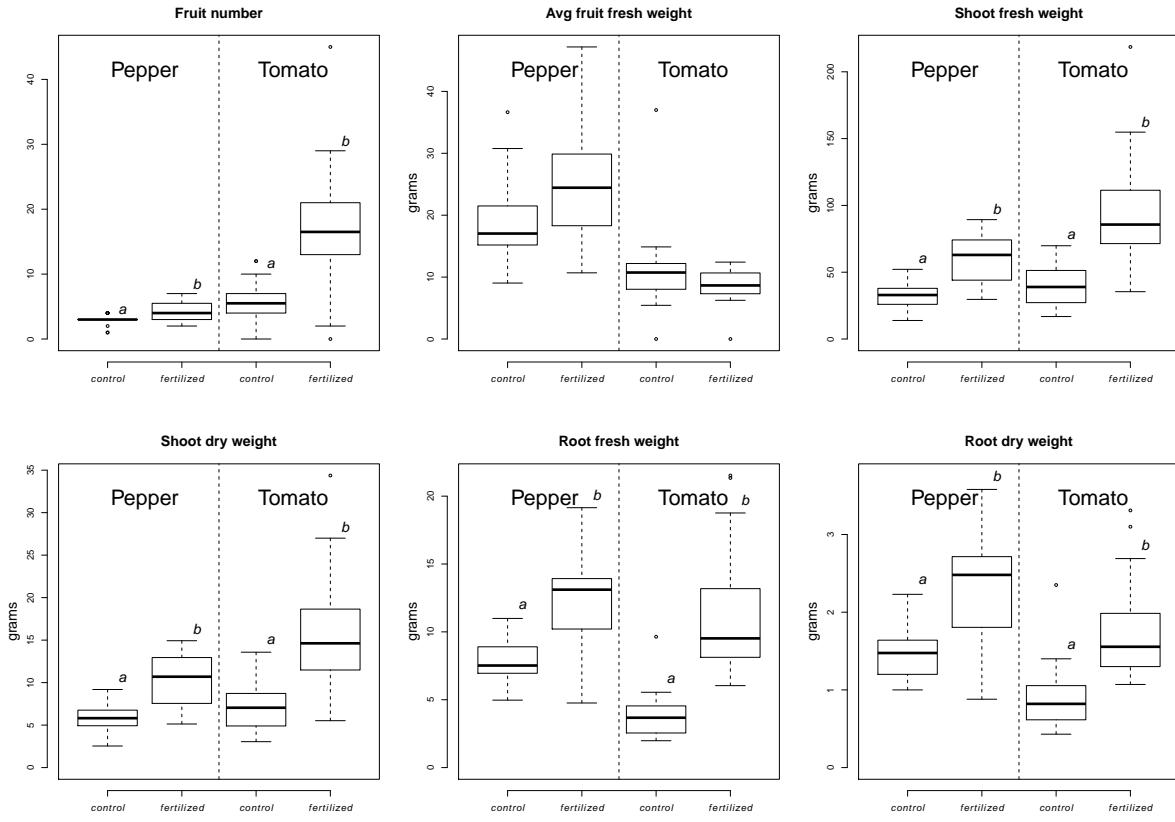


180
181 **Figure 2: photos of plant productivity. From top left to bottom right, fertilized pepper plants,**
182 **pepper roots, pepper fruits and tomato fruits and pictures to the left of the control plants.**

183

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

191



192

193 **Figure 3: measures of plant productivity.**

194

195

196 *Sequencing*

197 A total of 2.7 million paired-end raw reads were obtained for all samples combined (976,000 for
198 fungi-soil, 920,000 for fungi-root, 309,000 for bacteria-soil and 535,000 for bacteria-root, Table 2).

199 Note that sequencing samples were analysed separately for fungal-soil, fungal-root, bacterial-soil
200 and bacterial-root conditions. On average, 46,965 paired-end reads were obtained per sample,
201 and after quality filters were applied, including removing chimeras and paired-end reads were
202 merged, an average of 18,435 sequences remained. While 192 soil samples for fungi and bacteria,
203 and 92 root samples for fungi and bacteria were sequenced, three fungi-soil samples, 13 fungi-root
204 samples and two bacteria-root samples were removed because they had too few reads.

205

206 On average, 112 Amplicon Sequence Variants were identified per sample (average of 163 fungal-

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

213

214

Table 1: Sequencing and ASV summary

	fungi_soil	fungi_root	bacteria_soil	bacteria_root
Nb_samples	192	96	192	96
Nb_samples_trimmed	189	83	192	94
Nb_seq_sumX10e3	976	309	920	535
Nb_seq_mean	51381	32208	47907	56365
Nb_seq_mean_filtered	38045	14635	38287	46081
Nb_seq_mean_filt_merged	32014	13335	13780	41058
Nb_seq_mean_filt_merg_non_chimeras	24737	8505	12049	28451
ASV_persample	163	49	112	122
ASV_sum	6178	930	10120	3143
ASV_sum_trimmed	418	169	206	250

215

216

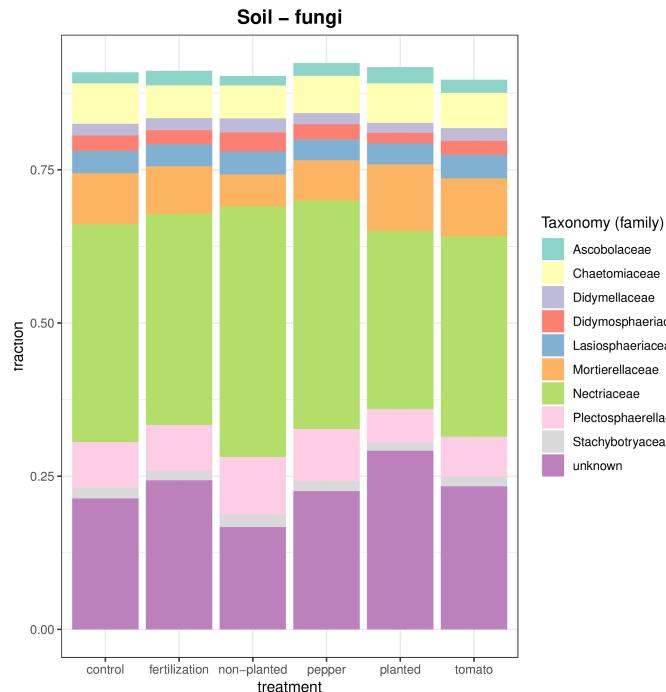
217 *Root & soil microbial and bacterial diversity*

218 We then analysed the whole community structure and report the relative abundance of taxa (fam-
219 ily) for the fungal-soil, fungal-root, bacteria-soil and bacteria-root conditions (Figure 4). Fungal
220 communities were dominated but Nectriaceae, both the root and soil samples. Bacterial root com-
221 munities were largely dominated by the class Oxyphotobacteria, but which could not be identified

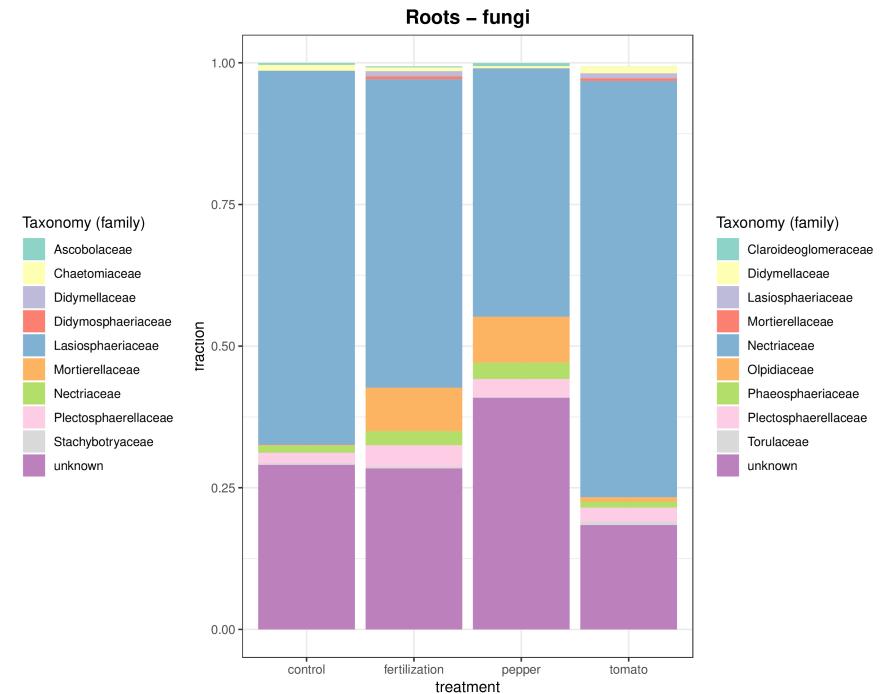
222 at the family level, while Bacillaceae dominated to a lesser extent the soil communities.

223

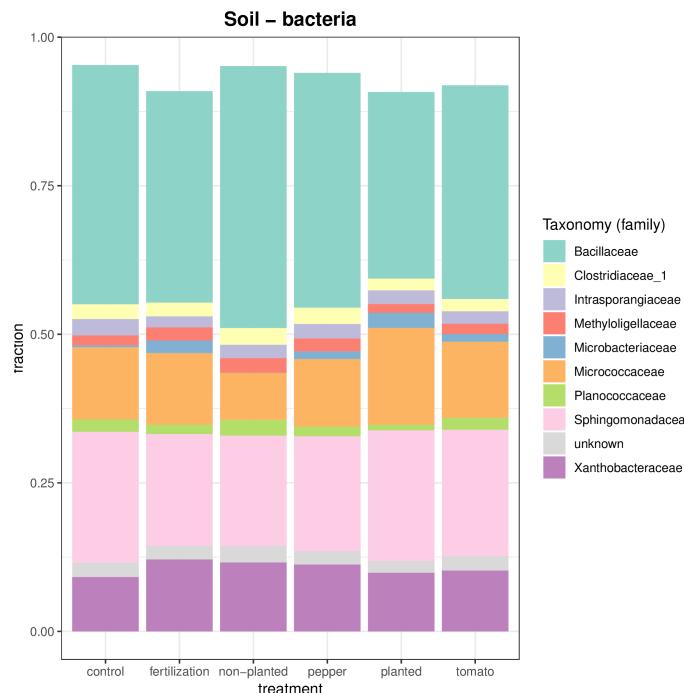
A



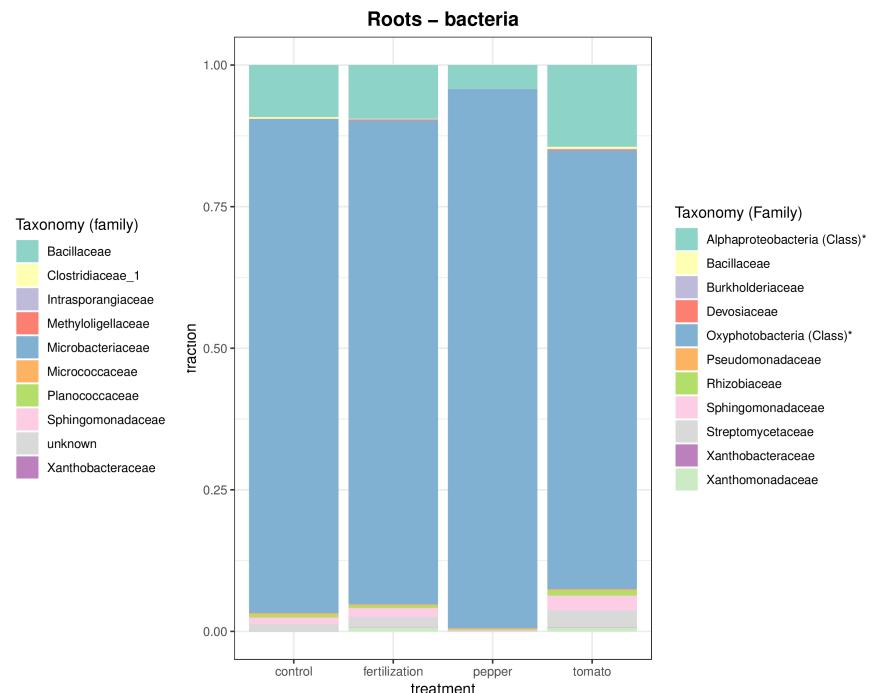
B



C



D



224

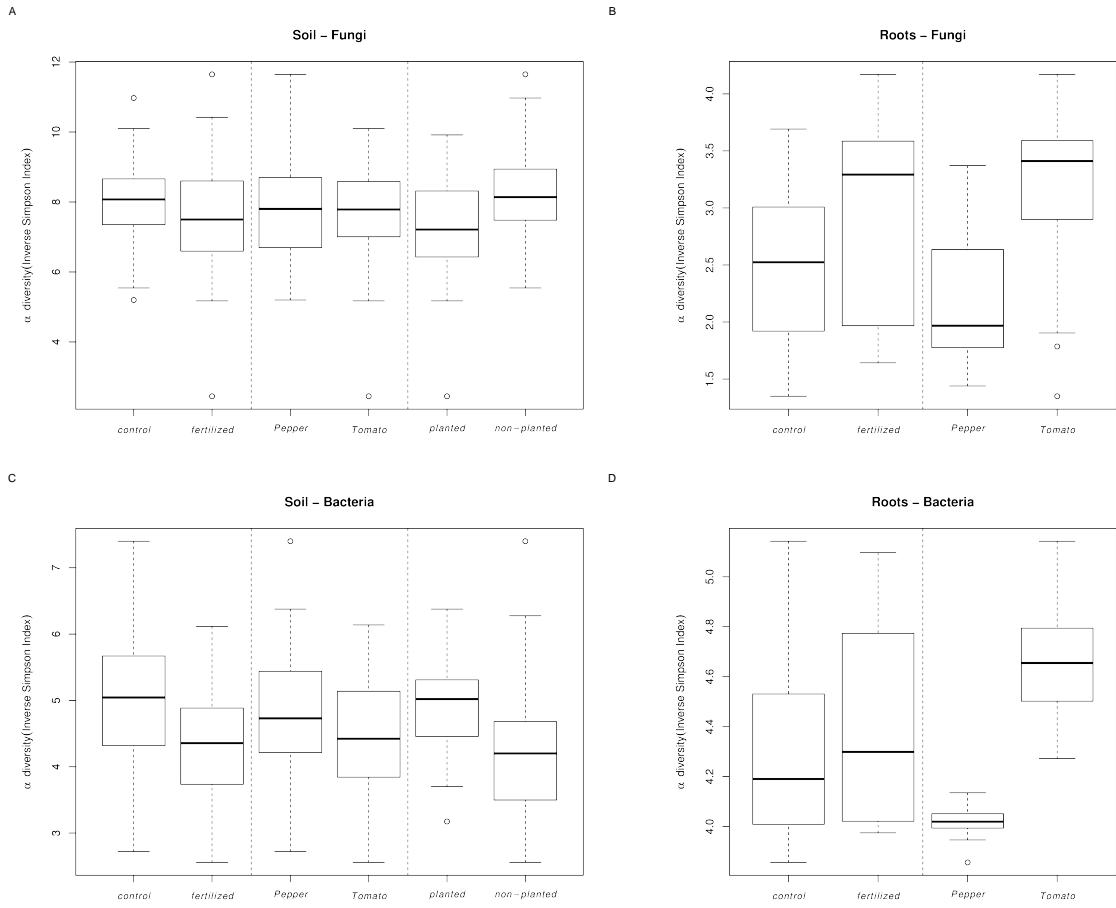
²²⁵ **Figure 4: Barplots.**

²²⁶

²²⁷

²²⁸ *Local (α -diversity)*

²²⁹ The diversity of each site (α -diversity) was calculated for each sample for each of the conditions
²³⁰ (fungi-soil, fungi-root, bacteria-soil and bacteria-root) and linear mixed effects models used to as-
²³¹ sess significance (see Figure 5). In soils samples, fungal diversity differed with respect to the fer-
²³² tilization ($F_{(1,161)}=14.35$, p -value<0.0001) and planting ($F_{(1,161)}=41.00$, p -value<0.0001) treatment,
²³³ but not the species ($F_{(1,161)}=0.13$, p -value=0.72). In root samples, fungal diversity differed with
²³⁴ respect to the fertilization treatment ($F_{(1,56)}=13.56$, p -value=0.001), and the species ($F_{(1,56)}=74.31$, p -
²³⁵ value=0.003). In soil samples, bacterial diversity differed with respect to the fertilization treatment
²³⁶ ($F_{(1,165)}=46.25$, p -value<0.0001), planting ($F_{(1,165)}=48.77$, p -value<0.0001) and species ($F_{(1,165)}=10.22$,
²³⁷ p -value=0.002). In root samples, bacterial diversity differed with respect to the fertilization treat-
²³⁸ ment ($F_{(1,67)}=16.48$, p -value=0.0001), and the species ($F_{(1,67)}=523.42$, p -value<0.0001).



239

240 **Figure 5: Barplots.**

241

242

243 *Differences in species composition among sites*

244 Using a PERMANOVA statistical framework, we identified that for all conditions, communities
 245 differed with respect to the fertilization treatment (Table 2). Soil fungal and bacterial communities
 246 differed the most according to whether the tray was planted, while root communities differed the
 247 most between tomato and pepper plants.

248

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

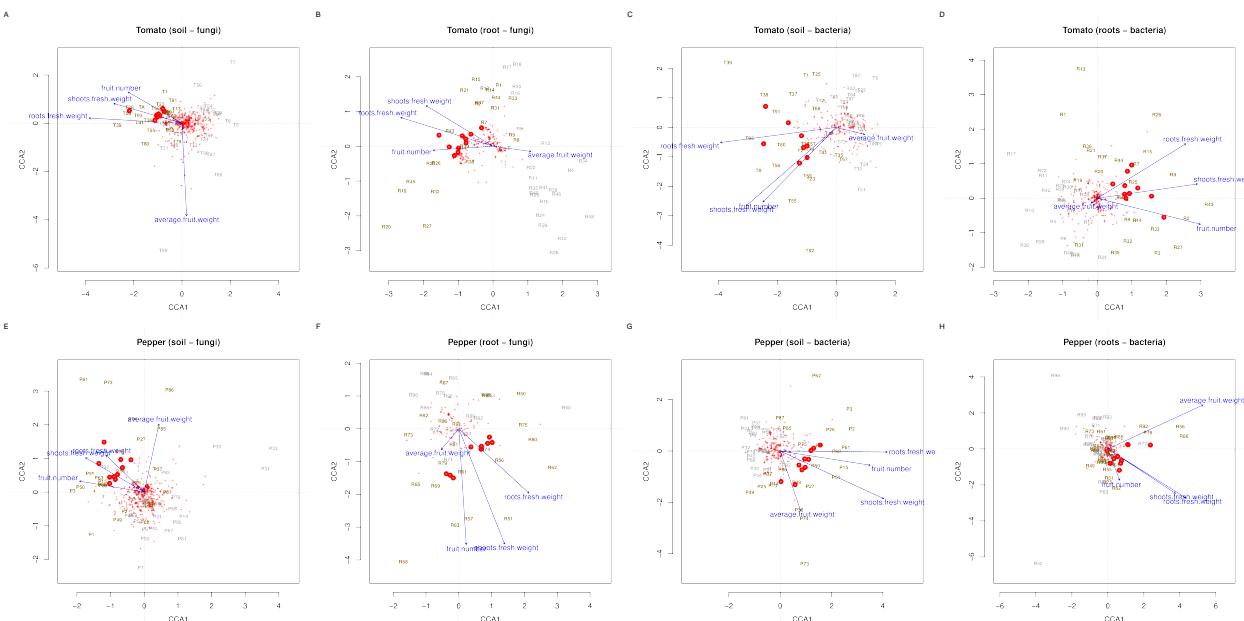
249 * R^2 [percentage of variance explained by the term in the model] and associated p-values

250

251 *Constrained ordinations and candidate ASVs*

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

257



258

259 **Figure 6: rda.**

260

261

262 Next, we identified, for each ordination, the ten ASVs which were most closely related to the
 263 three constraints which behave in a similar fashion (productivity measures of root fresh weight,
 264 shoots fresh weight and fruit number). These were considered as putative candidates which may
 265 be most positively impacted (increase presence of the candidate) by fertilization. We aligned the
 266 corresponding sequences for these eighty candidates (ten candidates * eight ordinations) ASVs in
 267 two separate alignments (one for fungi and one for bacterial ASVs), built distance matrices and
 268 plotted neighbouring joining trees. In fungi, we identified one cluster of ASVs taxonomically as-
 269 signed to *Olpidium brassicae* that forms the majority of ASVs most closely related to productivity.
 270 In addition, we identified seven closely related sequences closely related to productivity in all both
 271 species and both root and soil. Unfortunately, we could not assign taxonomy. SO WE BLASTED
 272 THESE SEQUENCES TO OBTAIN MORE INFO ABOUT WHAT IT IS.

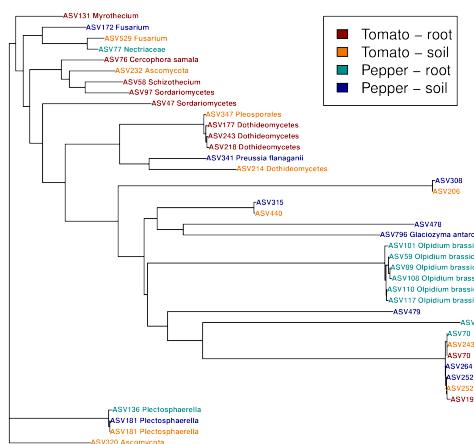
273 In bacteria, we identified a cluster of ten closely related sequences taxonomically assigned to
 274 "Chloroplast". In fact this is most likely the plant extract itself!!! Then we also have marine sps in
 275 the soil (ASV267:Ilumatobacteraceae), which may come from the extract itself...

276

277

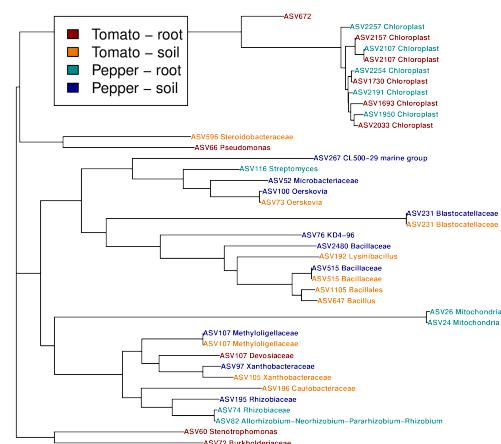
B

Fungi



A

Bacteria



278

279 **Figure 7: Candidate trees**

280

281

282 #DISCUSSION

283

284 #REFERENCE

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