

¹ **The effect of *Ascophyllum nodosum* extracts on tomato
2 and pepper plant productivity and root and soil
3 microbiome and fungal communities.**

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⁷ The abstract will be written here

⁸ *Keywords:* Stella Marris, 16S, ITS, microbial diversity, Illumina MiSeq

9 INTRODUCTION

10 The aim of this project was to develop a better understanding of the effects of *Ascophyllum no-*
11 *dosum* extracts on plant growth. In addition, we tested how the bacterial and fungal communities
12 responded to the additin of *A. nodosum* extracts.

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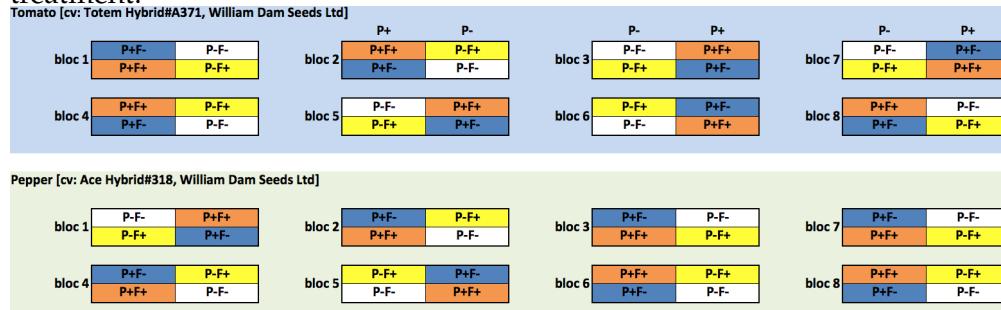
14 MATERIAL AND METHOD

15 Study design

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

22

23 For each species tested (Tomato - *Solanum lycopersicum* and Pepper - *Capsicum annuum*), a ran-
24 domized split block design (Figure 1) was used with four trays set up per block (eight blocks).
25 Half of the trays were fertilized (fertilization treatment) as described below. Half of the trays were
26 also planted with four replicate plants each, while the other trays were left bare. This allowed to
27 compare the fungal and bacteria soil communities with respect to the fertilization and planting
28 treatment.



30 Figure 1: experimental design

31

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

41

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

45

46

47 *Plant productivity*

48 At the end of the experiment, plant productivity was assessed by measuring four different traits
49 (fruit number, average fruit weight, shoots fresh weight, roots fresh weight) on three plants chosen
50 randomly per tray (for each treatment [fertilization/control], species [tomato/pepper] and block
51 [eight blocks]) for a total of 96 samples. In addition, both shoots and roots were dried in a 70 de-
52 grees drying oven, and dry weights were measured after 48hrs. Together, these traits are known
53 to represent well the plant overall productivity (ref.).

54

55

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

57 We collected the microbial and fungal communities from both soil and root samples. Amplicon
58 sequencing targeting 16S rRNA gene (bacteria) and ITS (fungi) was performed on both root and
59 soil samples. DNA was extracted from X g of plant root and Y g of soil samples using the Y DNA
60 Isolation Kit.

61

62 In order to target fungi, we used fungal primers ITS3_KYO2 (5'-ACACTGACGACATGGTTCTACAGATGAAGA
63 3') and ITS4_KYO3 (5'-TACGGTAGCAGAGACTGGTCTCTBTVCCKCTTCACTCG-3') to pro-
64 duce a final amplicon size of 430bp (Toju *et al.* 2012).

65

66 Bacterial primers 341F (5'-CCTACGGGNNGCWGCAG-3') and 805R (5'-GACTACCAGGGTATCTAAC-
67 3') producing a final amplicon size of ~464b and targetting specifically the bacterial V3-V4 re-
68 gion of the 16S ribosomal gene were chosen given that they has been used extensively in high-
69 throughput sequencing studies in a range of environments (Hugerth *et al.* 2014). This primer pair
70 was shown to be the least biased among 512 primer pairs evaluated in silico for bacterial amplifi-
71 cation (Klindworth *et al.* 2012).

72

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

77

78 *Bioinformatics* All bioinformatics, statistical, and graphic analyses further described were per-
79 formed in R 3.4.4 (R Core Team 2018) and detailed scripts are available here (https://github.com/seb951/Acadian_Seaplants).

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

87

88 First, sequence were trimmed following strict quality thresholds (see parameter details in the ac-
89 companying R pipeline). Following this, we applied the error model algorithm of dada2 which
90 incorporates quality information after filtering, unlike other OTU based methods. Then dereuplica-
91 tion, sample inference, merging of paired end reads and removal of chimera reads were performed

92 in order to obtain a sequence (ASVs) table of abundance per sample. Taxonomy was also assigned
93 using the Ribosomal Database Project (RDP) Naive Bayesian Classifier algorithm from Wang *et al.*
94 (2007). Depending on support (minimum bootstrap support of 80), we assigned taxonomy from
95 Kingdom to species. We used the silva database formatted for dada2 to infer bacterial taxa (Calla-
96 han 2018). We used the UNITE (2017) fasta release (including singletons) to infer fungal taxa after
97 formating it to the dada2 format using a custom R script. The dada2 pipeline was run on a mul-
98 tithreated (48 CPUs) computer infrastructure provided by Westgrid (<https://www.westgrid.ca/>)
99 [support/systems/cedar](#)) and Compute Canada (www.computecanada.ca). Note that the pipeline
100 was run separately for fungal root, fungal soil, bacteria soil and bacteria root samples given the
101 markedly different type of amplicons, taxa and error models of each dataset.

102

103 *Statistical analyses - Plant productivity*

104 We tested for the effect of species (tomato vs pepper), fertilization and their interaction on six
105 plant productivity measures (fruit number, average fruit weight, shoots fresh weight, roots fresh
106 weight, shoots dry weight, roots dry weight). We used linear mixed effect models (LMM) in the R
107 package NLME (Pinheiro *et al.* 2017), which are more appropriate than ANOVAs given the current
108 block design (blocks and replicates nested within a block were treated as random variables). All
109 six plant productivity measures were square root transformed in order to help satisfy the assump-
110 tion of normality of the residuals in the LMM statistical framework.

111

112

113 *Statistical analyses - microbial and fungal diversity*

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

122

123 We then conducted community-based analyses looking at the effect of the fertilization on the
124 abundance ASV taxa in the tomato and pepper experiments. To reduce the complexity of the
125 datasets, relative abundance of all taxa were calculated per family using dplyr (Wickham *et al.*
126 2015). Barplots were drawn using ggplot2 (Hadley 2016) to vizualize the communities. ASV
127 (*a*)-diversity was calculated for each sample using the inverse Simpson diversity index in the R
128 package VEGAN (Oksanen *et al.* 2013). The effect of treatment, species (and planting for soil
129 communities) were assessed using a linear mixed-effect (LMM) model in the R package NLME
130 (Pinheiro *et al.* 2017), given the unbalanced, replicated block design. Alpha diversity was log
131 transformed in order to help satisfy the assumption of normality of the residuals of the LMM.

132

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

138

139 We also performed constrained ordinations (cca) using Hellinger-transformed ASV abundance
140 data in VEGAN (Oksanen *et al.* 2013) to visually assess the grouping of samples, ASVs and
141 their association with productivity variables. Data were seperated by species, root/soil and taxa
142 (fungi/bacteria) givint a total of eight CCAs. Data were constrained based on four of the pro-
143 ductivity measures (fruit number, average fruits weight, shoots fresh weight, roots fresh weight).
144 We excluded the shoot & root dry weights as constraints to simplify the model given that they
145 were highly correlated with the fresh weighth already included as constraints ($r^2=0.98$ and 0.76
146 for shoot dry/freshweight and root dry/freshweights, respectively). We then identified the ten
147 ASVs most closely associated with fruit number, shoots fresh weight and roots fresh weight from
148 each constrained ordinations for a total of 40 fungal and 40 bacterial candidates ASVs. We then
149 plotted a phylogenetic tree for bacteria and fungi using ape (Paradis *et al.* 2004) and phangorn
150 (Schliep 2010) to see if identify candidate ASVs found under different experimental conditions
151 (soil/root,pepper/tomato).

¹⁵² A partition of the variation was also performed to assess how much of the variation was ex-
¹⁵³ plained by the soil and the vegetation characteristics.

154 **RESULTS**

155 *productivity* We tested the effect of the fertilization treatment on six productivity measures (fruit
156 number, average fruit weight, shoots fresh weight, roots fresh weight) for both tomato and pep-
157 pers. Visually, it appeared like there is a strongs correlation between fertilization and growth
158 (Figure 2).

159

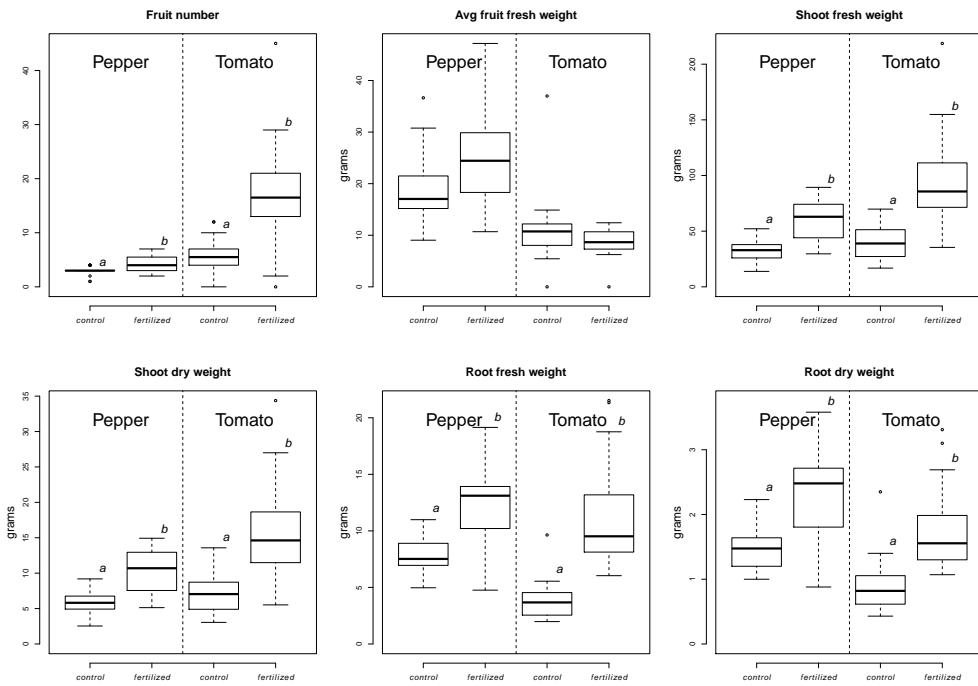


160 **Figure 2: photos of plant productivity. From top left to bottom right, fertilized pepper plants,
161 pepper roots, pepper fruits and tomato fruits and pictures to the left of the control plants.**

163

164 Statistically, all six productivity measures significantly differed according to species (Figure 3),
165 and five of those were significantly different according to the fertilization treatment. The only
166 exception was the average fruit weight which did not differ between fertilized and control plants
167 (LMM, $F = 1.27$, $p\text{-value}=0.26$). However the model did reveal a significant interaction between
168 treatment and plant ($F(1,69) = 9.6, p\text{-value}=0.0028$), such that when testing only the pepper plants,
169 the effect of fertilization on average fruit weight was significantly higher in the fertilized pepper
170 plants ($F(1,23) = 10.84, p\text{-value}=0.0032$).

171



172

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

174

175

176 *Sequencing* A total of 2.7 million paired-end raw reads were obtained for all samples combined
177 (976,000 for soil fungi, 920 for root fungi, 309 for soil bacteria and 535 for root bacteria, Table 2).

178 On average, 46,965 paired-end reads were obtained per sample, and after quality filtered were
179 applied, including removing chimeras and paired-end reads were merged, an average of 18,435
180 sequences remained. While we had sequenced 192 soil samples for fungi and bacteria, and 92 root
181 samples for fungi and bacteria, we removed three soil fungi samples, 13 root fungi samples and
182 two root bacterial samples because they had too few reads.

183

184 On average, 112 Amplicon Sequence Variants were identified per sample (average of 163 fungal
185 ASV in the soil, 112 bacterial ASV in the soil, 49 fungal ASV in roots, 122 bacterial ASV in roots).

186 Many of those were unique to one of a few samples (total number of 6178 fungal ASV in the soil,
187 10120 bacterial ASV in the soil, 930 fungal ASV in roots, 3143 bacterial ASV in roots). In fact, once
188 we removed ASVs that were found in fewer than 10% of the samples, we retained 418, 206, 169
189 and 250 ASV and which comprised 91%, 50%, 88% and 85% of all reads in the fungal soil, bacterial

190 soil, fungal roots and bacterial roots samples, respectively.

191

192

Table 1: Sequencing and ASV summary

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

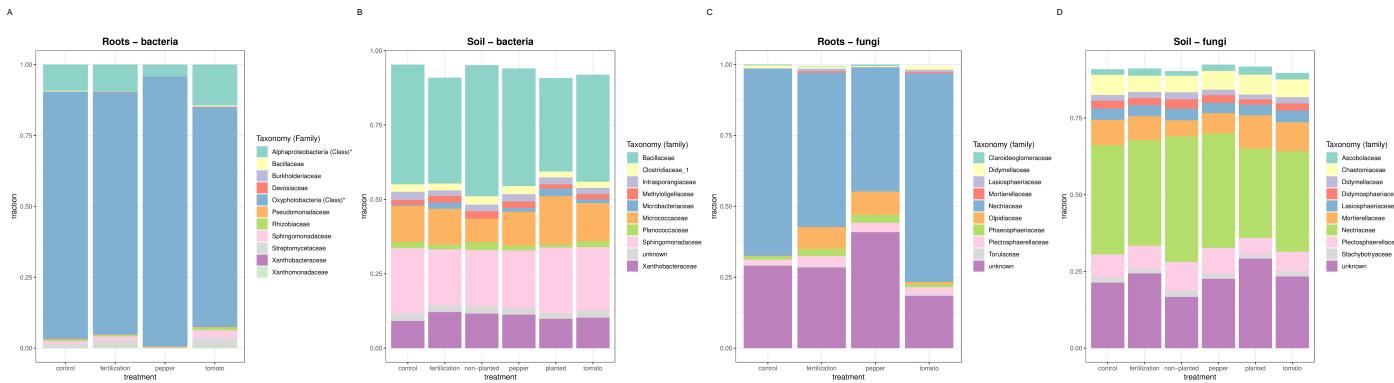
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194

195 *Root & soil microbial and bacterial diversity*

196 We then looked at the whole community structure and report the relative abundance of taxa (fam-
197 ily) for the root-bacteria, soil-bacteria, roots-fungi & soil-fungi conditions (Figure 4). In roots and
198 under all conditions, bacterial communities were largely dominated by Oxyphotobacteria (class)
199 which could not be identified at the family level, while Bacillaceae dominated to a lesser extent the
200 soil communities. With respect to fungi, Nectriaceae dominated both the root and soil samples.

201

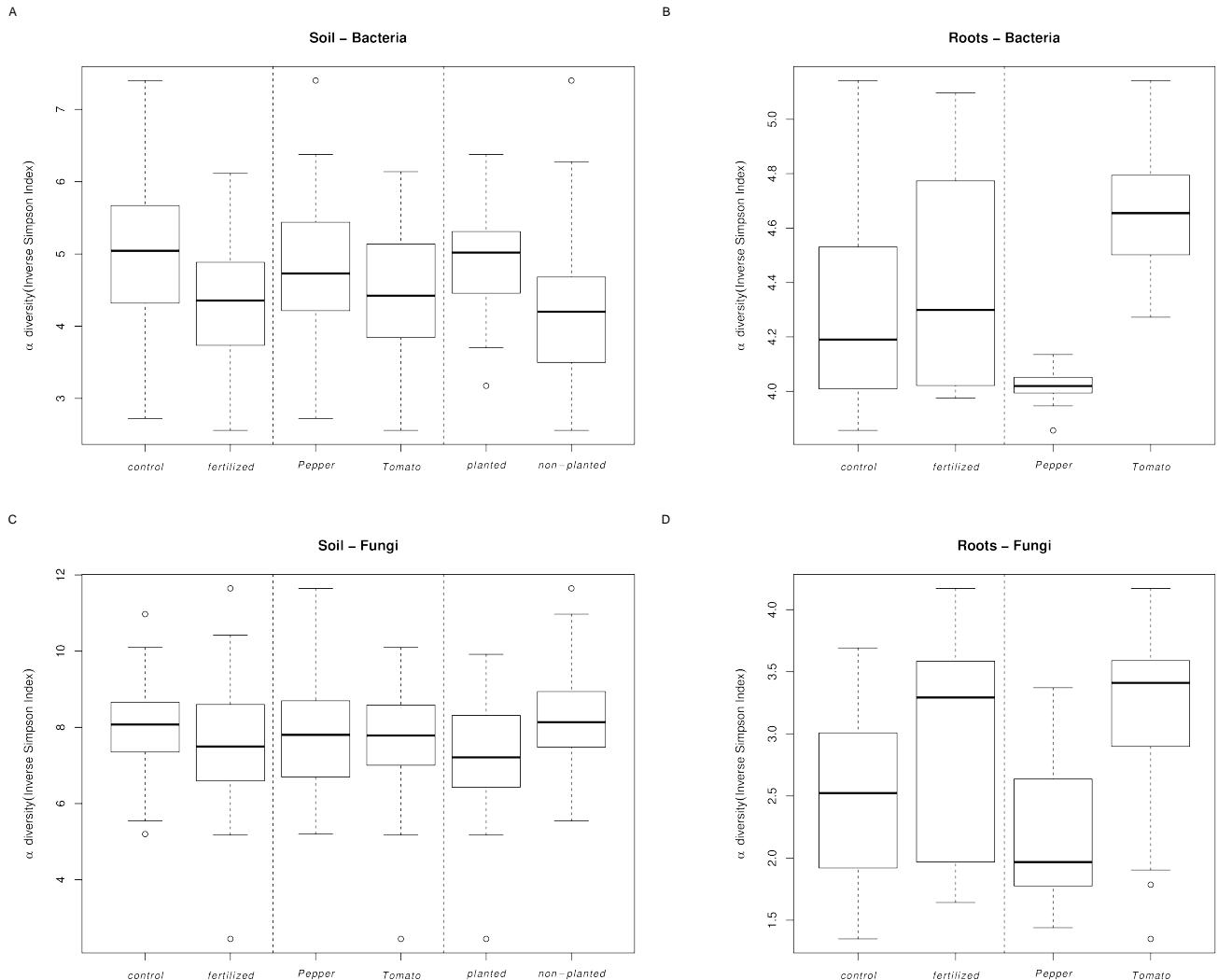


202

203 **Figure 4: Barplots.**204 *alpha diversity*

205 We then looked at alpha diversity

206



207

208 **Figure 5: Barplots.**

209 *permanova*

210 Communities differed among species and treatment

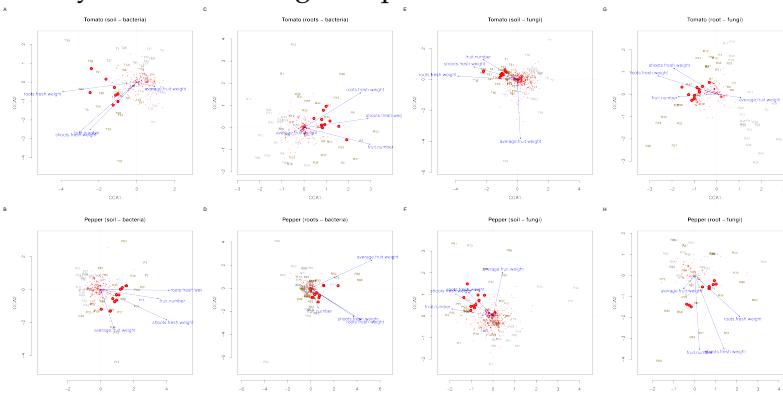
211 Lorem Ipsum...

Table 2: PERMANOVAs (R^2 and p -values)

	soil_fungi	root.fungi	soil_bact	root_bact
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

212 *constrained ordinations and candidate ASVs*

213 Relationships between environmental variables and prokaryotic communities. Canonical redundancy analysis indicated that distribution of phyla in the studied soils could be significantly explained by estimated nitrogen deposition,



216

217 **Figure 6: rda.**

218 Phylogenetic trees of candidate ASVs.

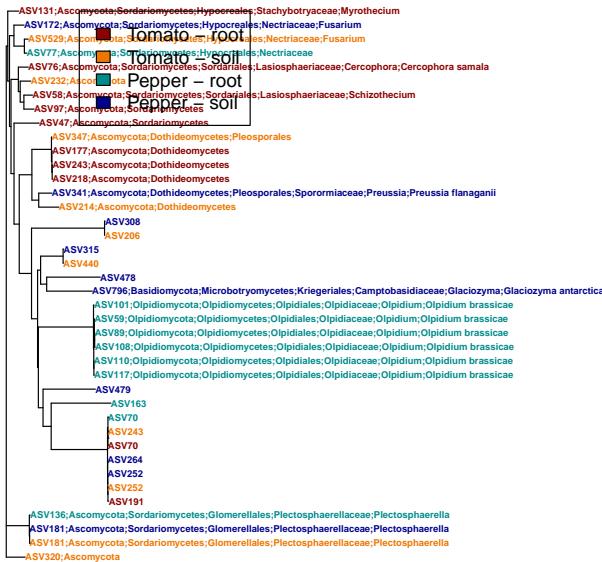
Bacteria



219

220 **Figure 7a: trees**

Fungi



221

222 **Figure 7b: trees**

223 DISCUSSION

224 **REFERENCE**

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