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### *Instantaneous responses of microbial communities to stress in soils pretreated with Mentha spicata essential oil and/or inoculated with arbuscular mycorrhizal fungus*

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**Instantaneous responses of microbial communities to stress  
in soils pre-treated with *Mentha spicata* essential oil and/or  
inoculated with AM fungus**

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Abstract:	The instantaneous response of a soil microbial community to a chemical stressor ( <i>Mentha spicata</i> essential oil) was studied post acclimation to the same chemical treatment at lower exposure. Acclimation involved repeated addition of small amounts of the essential oil weekly, for a period of a month whilst for the stress treatment a ten-fold exposure level was introduced. We also tested the role of AMF to the same stress exposure by pre-inoculating plant roots in the soil with the arbuscular mycorrhizal fungus (AMF) <i>Rhizophagous irregularis</i> . Three days after stress exposure, the structure of the soil microbial community was investigated plus the activities of six soil enzymes mainly related to N-cycle. The two pre-selected by AMF inoculation and acclimation soil microbial communities responded differently to the subsequent stress. Acclimation enhanced the biomass of G+ bacteria, fungi and micro-eukaryotes showing a priming effect of a low intensity stimulus when applied repeatedly, while AMF inoculation decreased the biomass of these microbial groups. The relative changes in microbial biomasses in jointly pre-treated samples were not different from the control, suggesting opposing effects of the two pre-treatments. On the contrary,

	<p>the jointly pre-treated samples responded to stress exposure by exhibiting increased activity of asparaginase and glutaminase and reduced activity of arylamidase. Finally, the relationship between enzyme activities and certain microbial ratios denotes that specific activities depended on the relative abundance of specific functional groups (e.g. G+ or G-) rather than on their biomass per se.</p>

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2 **Instantaneous responses** of microbial communities to stress in soils  
3 **pre-treated with *Mentha spicata* essential oil and/or inoculated with**  
4 **AM fungus**

5

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23

24 **Running head:** response of microbial communities to stress

25

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30 **Abstract**

31 The instantaneous response of a soil microbial community to a chemical stressor  
32 (*Mentha spicata* essential oil) was studied post acclimation to the same chemical  
33 treatment at lower exposure. Acclimation involved repeated addition of small  
34 amounts of the essential oil weekly, for a period of a month whilst for the stress  
35 treatment a ten-fold exposure level was introduced. We also tested the role of AMF to  
36 the same stress exposure by pre-inoculating plant roots in the soil with the arbuscular  
37 mycorrhizal fungus (AMF) *Rhizophagous irregularis*. Three days after stress  
38 exposure, the structure of the soil microbial community was investigated plus the  
39 activities of six soil enzymes mainly related to N-cycle. The two pre-selected by AMF  
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41 subsequent stress. Acclimation enhanced the biomass of G<sup>+</sup> bacteria, fungi and micro-  
42 eukaryotes showing a priming effect of a low intensity stimulus when applied  
43 repeatedly, while AMF inoculation decreased the biomass of these microbial groups.  
44 The relative changes in microbial biomasses in jointly pre-treated samples were not  
45 different from the control, suggesting opposing effects of the two pre-treatments. On  
46 the contrary, the jointly pre-treated samples responded to stress exposure by  
47 exhibiting increased activity of asparaginase and glutaminase and reduced activity of  
48 arylamidase. Finally, the relationship between enzyme activities and certain microbial  
49 ratios denotes that specific activities depended on the relative abundance of specific  
50 functional groups (e.g. G<sup>+</sup> or G<sup>-</sup>) rather than on their biomass *per se*.

51

52 **Keywords:** *Rhizophagous irregularis*, PLFAs, soil enzymes, soil ecology,  
53 acclimation

54

55

56 **Introduction**

57 Soil microbes can be exposed to rapid alterations in soil conditions including pH,  
58 water content, organic C, N or available P, salinity and oxygen concentration,  
59 influencing their physiology or survival. Schimel et al. (2007) defined stress as the  
60 factor that induces changes in the function and survival of organisms. Most studies  
61 examining the consequences of stresses focus **at the species level** (Begley, Gaham, &  
62 Hill, 2003; Leyer & Johnson, 1993). Microbes are frequently exposed to stress but  
63 their responses must be evaluated at the community level (Rillig, Rolf, Tietjen, Wehner,  
64 & Andrae-Linares, 2015), since different microbial strains aggregate and form colonies  
65 (Ekschmitt, Liu, Vetter, Fox, & Wolters, 2005). At the community level, the response to  
66 an intensive stimulus is less predictable and complex, since the effect of stress  
67 depends not only on the species-specific resistance but also on the type of interactions  
68 among the members of the community (competition, synergy, allelopathy, prey-  
69 predator relations) (Fraterrigo & Rusak, 2008; Karakoç, Singer, Johst, Harms, &  
70 Chatzinotas, 2017). Existing evidence suggests that the soil microbial community  
71 responds differently to various types of stress because microbiota behaviour varies in  
72 terms of species mortality and the development of the various microbial species  
73 (Gibbons et al., 2016) and in terms of differential energy cost derived by the response  
74 of various strains to stress (Rillig et al., 2015). Furthermore, the resistance of the  
75 micro-organisms to stress factors is modulated by their previous exposure to a  
76 stimulus of the same or different nature but of lower intensity. Cells exposed to a mild  
77 primary stress could **even be more** resistant to subsequent severe secondary doses of  
78 the same stressors and show resistance to other stresses (Rillig et al., 2015).  
79 Resistance to stress at the microbial community level is an attribute of a stabilized

80 community (Święciło & Zych-Wężyk, 2013; Tardy et al., 2014) that can maintain  
81 biochemical transformations in soil.

82 In this study we examined the response of soil microbial community and enzymes  
83 to stress exposure imposed by the application of high dose of *Mentha spicata* essential  
84 oil in the rhizosphere soil of tomato plants grown under three different treatments (i)  
85 inoculation of tomato plant roots with the arbuscular mycorrhizal fungus  
86 *Rhizophagous irregularis* (ii) repeated application of low doses of *M. spicata* oil in  
87 the rhizosphere soil and (iii) application of the both treatments together. We focused  
88 on instantaneous responses to stress (3 days after stress application) since the  
89 microbial responses to such intervention can be rapid (Lehmann, Crombie, & Singer,  
90 2008).

91 Essential oils are constituents of aromatic plants that are common in the  
92 Mediterranean environment. The incorporation of essential oils or their major  
93 constituents into soils has stimulatory effects on bacterial populations (Vokou,  
94 Chalkos, Karamanlidou, & Yiangou, 2002), depresses specific fungal populations  
95 (Hassiotis & Dina, 2011; Kadoglidou et al., 2011) and can stimulate soil respiration  
96 (Vokou & Liotiri, 1999). Therefore, essential oils by acting selectively on members of  
97 the community may influence microbial community profiles (Lehmann et al., 2008).  
98 For example, the leaves and spearmint leaf extract (*Mentha spicata*) are rich in  
99 flavonoids (Naidu, Ismail, Yeng, Sasidharan, & Kumar 2012) and monoterpenes  
100 (Chowdhury, Nandi, Uddin, & Rahman, 2007). Several monoterpenes act on cell  
101 membranes by affecting lipid fraction of plasma membrane, causing intracellular  
102 membrane leakage (Trombetta et al., 2005). Also, monoterpenes could affect the  
103 respiratory enzymes of fungi (Cox et al., 2000).

104 **Importantly**, colonization of roots by arbuscular mycorrhizal fungi (AMF) **at the**  
105 **same time affects** the quality and quantity of host plant root exudates and the structure  
106 of the microbial communities in the rhizosphere (Tahat & Sijam, 2012). Interactions  
107 developed between AMF and free-living microbes include the binding of bacteria to  
108 the fungal spores, the production of volatiles by free-living bacteria involved in the  
109 degradation of fungal cellular walls and the growth of specialized bacteria that  
110 promote the activity and development of AM fungi (Miransari, 2011). Due to the  
111 multiplicity of the interactions between **rhizosphere microbes and AMF**, the latter  
112 have been employed as biofertilizers (Bona et al., 2015; Lioussanne, Perreault,  
113 Jolicoeur, & St-Arnaud, 2010).

114 According to **previous work** by Stamou et al. (2017), the two different treatments  
115 (AMF inoculation or application of low doses of *M. spicata* oil) acted selectively on  
116 members of the soil microbial community in a diverging way. **We hypothesized that**  
117 **“selected” microbial communities (via AMF inoculation and / or *M. spicata* oil**  
118 **acclimation) will respond differently when exposed to a subsequent stress episode**  
119 **(higher level exposure to *M. spicata* oil).** Furthermore, we examined under which  
120 conditions could there be a synchronization between the microbial community  
121 structure and the soil functionality as this described by enzymatic activities.

122

## 123 **Materials and Methods**

### 124 **Experimental Design**

125 Tomato seedlings (*Lycopersicon esculentum* var. *Ace*) from sterilized seeds, were  
126 cultivated hydroponically over three weeks and transplanted into surface sterilised (2  
127 L volume) pots. These were filled with a sterilized soil sand mixture in a ratio 1:1  
128 w/w (1500 g in each pot). Soil was an acid (pH 5) sandy loam one. The concentration



129 of organic C and nutrients before sterilization was C 16200 mg/Kg, N 960 mg/Kg,  
130 Pextr 21 mg/Kg, K 1.62 mg/Kg, Ca 1.17 mg/Kg, Mg 0.18 mg/Kg and Na 0.136  
131 mg/Kg. This chemical composition ensured the successful colonization of plants with  
132 AMF. To eradicate indigenous AMF and other soil borne biota, the soil-sand mixture  
133 was sterilized by autoclaving (4 h at 120 °C).

134 For half of all pots (n=9), the roots of tomato seedlings were inoculated with 10 g  
135 of *Rhizophagus irregularis* inoculum whilst the other half (n=9) were non-  
136 inoculated. The inoculum consisting of spores and hyphal fragments of *R. irregularis*,  
137 provided by the Energy and Resource Institute, India at a concentration of 1000  
138 propagules per gram of inoculum clay powder. Prior to the experiment, to test the  
139 quality of the inoculum, it was applied in 5 mixed pot cultures of *Plantago*  
140 *lanceolata*, *Dactylis glomerata*, and *Trifolium repens*, and the colonization rate was  
141 estimated 40, 60 and 80 days after the application of the inoculum. In all cases the  
142 root colonization rate was >80%, 40 days after the application.

143 Ten days after root inoculation, we reintroduced the pre-existing bacterial  
144 community of the soil, by adding a bacterial inoculum prepared from the soil initially  
145 collected. For the preparation of the bacterial inoculum, 10 g of the initially collected  
146 soil were mixed with 50 ml of deionized water, the soil suspension was filtered  
147 through a 21 µm sieve, and 10 ml of it was added to each pot near the rhizosphere  
148 zone. The 10-days delay between AMF inoculation and the addition of bacterial  
149 inoculum was necessary for plant-fungus interactions to take place since microbiota  
150 could reduce the extent of AMF root colonization (Stamou et al., 2017).

151 Thirty days after inoculation, inoculated pots (n=3) were treated with *M. spicata*  
152 essential oil at a weekly rate of 1.33 ml per pot over a period of one month  
153 representing acclimation, with further pots (n=3) untreated. The quantity of essential

154 oil introduced near the **plant rhizosphere was** in accordance with previous studies  
155 (Vokou & Margaris, 1988; Vokou et al., 2002). The oil was supplied by Etherio,  
156 Research and Commerce, Eratera, Greece and **was** pure essential oil produced after  
157 distillation of *M. spicata* plants. The major compounds of *M. spicata* oil were carvone  
158 63.9% and limonene 13.3% followed by 1,8 cineole,  $\beta$ -pinene, myrcene and  $\alpha$ -pinene  
159 in percentages 7.1, 2.8, 2.4 and 1.4%, respectively (Stamou et al., 2017). Following  
160 one month acclimation, a **'stress event'** was simulated by applying a tenfold amount  
161 of *M. spicata* oil (13.3 ml per pot) in six out of the nine inoculated pots. The same  
162 treatment regime was applied to the non-inoculated pots.

163 There were four treatments (**two AMF** inoculation levels (+, -) x **two** acclimation  
164 levels (+, -) with three replicates per treatment giving a total of 12 pots in a  
165 randomized block design: (i) inoculated-acclimated-stressed pots (+AMF+AC+ST),  
166 (ii) inoculated-non acclimated-stressed pots (+AMF-AC+ST), (iii) non-inoculated-  
167 acclimated-stressed pots (-AMF+AC+ST), (iv) only stressed pots (+ST; control).  
168 **From comparison of** (i) and (iv) the **combined** effect of inoculation and acclimation  
169 **was** revealed. **Next,** by comparing (ii) to (iv) and (iii) to (iv) the effect of inoculation  
170 and acclimation **were** examined, respectively. Moreover, in order to have an estimate  
171 of root colonization in non-stressed samples, **inoculated only** (+AMF) and non-  
172 inoculated pots (-AMF) were used as control treatments **involving** three replicates  
173 each (Stamou et al., 2017).

174 Experiments were conducted in a greenhouse under natural light conditions for a  
175 two-month period (from mid-June to mid-August). During the period of plant growth,  
176 the day temperature ranged from 28-37 °C and the night temperature from 20-27 °C.  
177 Plants were watered daily to maintain 60% of soil water holding capacity and no  
178 fertilizer was applied. Destructive sampling was conducted 3 days after the initiation

179 of high dose *M. spicata* essential oil stress.

180

### 181 Soil sampling

182 From each pot we collected six subsamples (2 cm diameter x 15 cm height) at a  
183 distance of 5 cm in a circle around the plant. Subsamples were bulked to get one  
184 composite soil sample per pot. These composite samples were analysed for the  
185 concentration of phospholipid fatty acids (PLFAs) and enzyme activities. Each  
186 composite sample was sieved through a 2 mm mesh to remove roots and organic  
187 debris and from 1 mm mesh to keep sand particles away. Samples were subsequently  
188 stored at a constant temperature at 4°C until used, within the same week.

189

### 190 AMF analysis

191 Plant roots were cleaned of any soil or sand particles by use of an ultrasonic bath at  
192 50Hz. Prior to estimating the colonization rate by AMF, all roots were immersed in  
193 50% (v/v) ethanol at 5 °C. For estimation of AMF colonization, 40% of the root  
194 samples were randomly used. Root samples were rinsed with distilled water, stained  
195 with 0.05% (w/v) Trypan Blue in acidic glycerol overnight and then incubated in 10%  
196 (v/v) KOH, at room temperature, for 24 h (Koske & Gemma, 1989). The method was  
197 originally proposed by Koske and Gemma (1989) and was modified by Orfanoudakis,  
198 Wheeler, and Hooker (2010). The stained samples were examined under a compound  
199 microscope (Nikon E6000 Eclipse) applying x40 total magnification, and the AMF  
200 percentage colonization was evaluated according to Trouvelot, Kough, and  
201 Gianinazzi-Pearson (1986).

202

## 203 Enzymatic activity assays

204 The activities of six soil enzymes were studied. These were N-acetyl-glucosaminidase  
205 (NAG), acid phosphatase, urease, asparaginase, glutaminase and arylamidase. N-  
206 acetyl-glucosaminidase (NAG) and acid phosphatase activities were determined  
207 according to the procedures of Allison and Jastrow (2006), as these were modified in  
208 order to be applicable for 96-well microplates. Approximately 1-2 g fresh soil  
209 (equivalent to 0.5 g dry weight) were added in 60 ml of 50 mM sodium acetate buffer,  
210 pH 5, and homogenized in a blender for 1min. Then, 50  $\mu$ L of homogenized soil  
211 slurry were combined with 150  $\mu$ L substrate solution and incubated for 3 h (NAG) or  
212 1 h (acid phosphatase) at 21 °C under constant shaking. Substrate solutions were 2  
213 mM p-nitrophenyl- $\beta$ -N-acetylglucosaminide for NAG and 5 mM p-nitrophenyl-  
214 phosphate for phosphatase, all in acetate buffer. After incubation, 100 mL of the  
215 slurry-substrate supernatant (without soil particles) were carefully transferred to  
216 another microplate for colorimetric determination of product concentrations. The *p*-  
217 nitrophenol (pNP) reaction product from the phosphatase and NAG assays was  
218 measured at 405 nm, after addition of sodium hydroxide. Eight replicates were run per  
219 sample; in each case, we included appropriate controls to estimate the background  
220 absorbance of the substrate and homogenate. The activity of the two enzymes is  
221 presented in units of  $\mu$ mol pNP g<sup>-1</sup> dry soil h<sup>-1</sup>.

222 Urease activity was determined according to the methods of Sinsabaugh, Reynolds,  
223 and Long (2000). The microplate configuration was similar to that described for the  
224 NAG assay. The concentration of urea in the assay wells was 20 mM. The plates were  
225 incubated at 20 °C for approximately 18 h. Ammonium released by the reaction was  
226 quantified using colorimetric salicylate and cyanurate reagent packages from Hach.

227 Urease activity was measured spectrophotometrically at 610 nm. Activity is expressed  
228 as micromoles of ammonium released per hour per g soil ( $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ ).

229 The activities of asparaginase and glutaminase were determined according to the  
230 methods of Tabatabai (1994). Briefly, the methods were based on the determination of  
231  $\text{NH}_4^+$  released when soil is incubated at 37 °C for 2 h with 0.1 M tris-hydroxymethyl-  
232 aminomethane (THAM) buffer, toluene and L-asparagine or L-glutamine for  
233 asparaginase and glutaminase, respectively. The  $\text{NH}_4^+$  released was determined by  
234 treating the incubated soil sample with 2 M KCl containing  $\text{Ag}_2\text{SO}_4$  (to stop the  
235 enzymatic activity) followed by steam distillation of an aliquot of the resulting soil  
236 suspension with MgO. The activities of these enzymes were assayed on <2 mm field-  
237 moist samples, at the optimal pH value, in duplicates and one control, and are  
238 expressed on a moisture-free basis. Moisture was determined after drying at 105 °C  
239 for 24 h.

240 Arylamidase activity was evaluated according to the method of Acosta-Martinez  
241 and Tabatabai (2000). One g air-dried soil was incubated at 37 °C for 1 h with the  
242 substrate L-leucine- $\beta$ -naphthylamide in THAM buffer (0.1 M, pH = 8.0). The reaction  
243 was stopped with ethanol and the product  $\beta$ -naphthylamide was measured  
244 colorimetrically at 540 nm after its reaction with *p*-dimethylamino-cinnamaldehyde.

245

#### 246 Phospholipid fatty acid analysis

247 Extraction and analysis of phospholipids (PLFAs) was performed within one week of  
248 harvesting. Briefly, this involved extraction of lipids, separation of phospholipids by  
249 column chromatography, methylation of esterified fatty acids in the phospholipid  
250 fraction, chromatographic separation and identification of the main components on a  
251 Trace GC ultra gas chromatograph (ThermoFinnigan, San Jose, CA) coupled with a

252 Trace ISQ mass spectrometry detector, a split–splitless injector, and an Xcalibur MS  
253 platform. Quantification of each fatty acid (in nmol g<sup>-1</sup>) was achieved by one point  
254 calibration against the GC response of the internal standard 19:0 methyl ester. Under  
255 the above conditions the GC response to 19:0 methyl ester is linear in the range of 25–  
256 200 µg ml<sup>-1</sup>, with acceptable recoveries (Spyrou, Karpouzas, & Menkissoglu-  
257 Spiroudi, 2009).

258 The total amount of PLFAs was used to account for the total microbial biomass.  
259 Overall, 22 fatty acid methyl esters were identified and considered for further analysis,  
260 including the internal standard 19:0. These are i-15:0, a15:0, 15:0, i16:0, i17:0 which  
261 were indicators of Gram<sup>+</sup> bacteria (McKinley, Peacock, & White, 2005; Myers, Zak,  
262 White, & Peacock, 2001; Zak et al., 1996), the bacteria indicators 16:0, 17:0 (Rillig,  
263 Mummey, Ramsey, Klironomos, & Gannon, 2006), the Gram<sup>-</sup> bacteria indicator  
264 16:1 $\omega$ 9c (Zak et al., 1996) and the indicators of actinomycetes 10Me16:0, 10Me17:0,  
265 10Me18:0 (Frostegård, Tunlid, & Bååth, 1993; White, Stair, & Ringelberg, 1996). All  
266 these were considered to be of bacterial origin only and were chosen to represent  
267 bacterial biomass. The 18:1 $\omega$ 9c and 18:2 $\omega$ 9,12 fatty acids were used as indicators of  
268 fungal biomass (Zak et al., 1996; Rillig et al., 2006) and the fatty acid 16:1 $\omega$ 5 was  
269 used as indicator of AM fungi and specifically of viable hyphal biomass because the  
270 fungal storage reserves such as spores, vesicles and propagules are represented by  
271 neutral 16:1 $\omega$ 5 (Olsson & Johansen, 2000). The PLFAs 20:0, 22:0, 23:0, 24:0 were  
272 considered as indicators of microeukaryotes (algae, protozoa, nematodes; (Smith et  
273 al., 1986). Finally the fatty acids 17:1, 18:0 and 14:0 were mainly of microbial origin.  
274 Moreover, the ratios Gram<sup>+</sup>/Gram<sup>-</sup> (G<sup>+</sup>/G<sup>-</sup>), fungi/bacteria (F/B), iso/anteiso (Iso/Ant)  
275 and Saturated/Unsaturated (Sat/Unsat) were estimated. Iso biomass is equal to the

276 sum of i15:0, i16:0 and i17:0 biomasses, while anteiso was represented by the  
277 biomass of a15:0.

278

279 Data analyses

280 To estimate the changes in biomass and enzyme values in pre-treated and stress  
281 exposed samples (inoculated or acclimated with oil or jointly treated) in relation to a  
282 control (only stress applied), we used the following equation (Rivest, Pawuette,  
283 Shipley, Reich, & Messier, 2015)

284 
$$\% \text{ relative change} = -100 * ((Co - So) / Co)$$

285 where Co is the variable's value in the control sample measured 3 days after stress  
286 application and So is the corresponding value in the treated sample measured at the  
287 same time point. A value of zero indicated no difference between the treated and  
288 control samples, negative changes indicated decreasing values and positive changes  
289 were indicative of higher values in treated samples compared to control.

290 To examine the effect of pre-treatments on the % relative change, we applied one-  
291 way analysis of variance on biomass and enzyme variables by Generalized Linear  
292 Models (GLM) (Distribution: Normal, Link function: Identity). In all cases, the  
293 significance of the relative changes was estimated in relation to the control.

294 To quantify causal relationships between predictors and response variables, we  
295 performed a Partial Least Square (PLS) analysis using the NIPALS/PLS regression  
296 algorithm of Statistica 7.0. PLS can be used instead of Multiple Regression but is best  
297 applied in cases where the number of predictors is large and where a high possibility  
298 of collinearity exists (Tobias n.d.). Specifically, we determined the extent to which the  
299 enzymatic profile (arylamidase, asparaginase, glutaminase, NAG, acid phosphatase,  
300 urease), could be predicted given the AMF inoculation, acclimation, stress, the

301 biomass of certain guilds and **the** structure of the microbial community represented by  
302 the various ratios. The algorithm extracted one component at a time and estimated the  
303 corresponding fraction of the explained variation, in particular  $R^2X$  is the variability in  
304 the predictor variables and  $R^2Y$  the variability in the set of the response variables.  
305 Prior to analysis the variables **were** rescaled in the range **of** 0-1 to ensure that the  
306 criterion for choosing successive factors **was** based on how much variation they  
307 explained **ed** (SAS/STAT (R) 9.22 User's Guide).

308 All analyses were performed by the STATISTICA 7.0 package (Statsoft, Tulsa,  
309 USA).

310

## 311 **Results**

312 The mean percentage colonization of tomato roots by the AM fungus was  $0.41 \pm 0.05$   
313 in inoculated-acclimated and stressed samples,  $7.43 \pm 2.25$  in inoculated and stressed  
314 samples and  $12.27 \pm 3.53$  in samples inoculated only. There was a tendency for a lower  
315 percentage of colonization in samples where oil was added either as pre-treatment or  
316 as a stress factor. In non-inoculated samples the colonization was  $<0.03\%$ .

317 Most microbial groups exhibited similar pattern of response to stress. Their relative  
318 changes were affected significantly by **AMF** inoculation and acclimation *per se* but  
319 not by their joint **application** (Table 1). This holds for the changes in total microbial  
320 biomass, the biomass of  $G^+$ , micro-eukaryotes and fungi. Changes in  $G^-$  bacteria were  
321 affected only by AMF. As shown in Fig. 1, AMF had a negative influence on relative  
322 changes whilst the effect of acclimation was positive. The biomass of most microbes  
323 increased significantly in stressed samples that **had** been acclimated (except  $G^-$   
324 bacterial biomass) while decreased in AMF inoculated-stressed samples. **However,**  
325 **there were no significant changes in actinomycetes observed.**



326 AMF inoculation positively and significantly affected the response of arylamidase,  
327 asparaginase, glutaminase and NAG to stress (Table 1, Fig. 2). Acclimation also  
328 induced positive relative changes in asparaginase and glutaminase and negative  
329 effects in arylamidase and urease. The relative changes in samples treated jointly with  
330 inoculation and acclimation were positive for asparaginase, glutaminase and negative  
331 for arylamidase. Phosphatase response to stress was unaffected by any type of pre-  
332 treatments.

333 The configurations of both the predictor and response variables on a PLS bi-plot is  
334 presented in Fig. 3. The percentage of variability in the predictive variables  
335 (inoculation, acclimation, stress and microbial community biomasses and the ratios  
336 among microbial groups) accounted for 56% by the two first components while the  
337 corresponding variability for enzymes was 46%. There was a clear discrimination of  
338 the treatments in relation to both axes. In relation to the first axis, samples were  
339 separated due to acclimation while in relation to second axis distinction was due to  
340 AMF inoculation. Acclimated samples (-AMF+AC) were characterized by high  
341 biomass values of all microbial groups, inoculated (+AMF-ACL) by high activity of  
342 arylamidase. Samples that had been exposed to inoculation and acclimation were  
343 separated due to the high values of microbial ratios and enzymes activity.  
344 Glutaminase NAG, and asparaginase, were related to G<sup>+</sup>/G<sup>-</sup>, Iso/Ant and to a lesser  
345 extent Sat/Unsat ratios. Moreover, the activity of acid phosphatase, urease and  
346 arylamidase was unrelated to the biomass and the structure of the community.

347

## 348 Discussion

349 Microbial community structure and enzyme activity

350 This study examined the instantaneous responses of pre-treated soil microbial  
351 communities to stress. The pre-treatments included inoculation of tomato plants with  
352 AMF inoculum or acclimation of soil with a low dose of *M. spicata* oil or both. Stress  
353 involved exposure to *M. spicata* oil at levels tenfold higher than the acclimation  
354 treatment.

355 As a response to stress, acclimation induced significant stimulatory effects on micro-  
356 eukaryotes, total microbial biomass, the biomass of G<sup>+</sup> bacteria and fungi. Micro-  
357 eukaryotes are grazers of microbial biomass, so the increase of their biomass and that  
358 of total microbial biomass were expected. The simultaneous increase of both groups  
359 may related to high rates of nutrient turnover due to predation, consequently  
360 supporting higher microbial biomasses in acclimated and stressed samples (Fig. 3).  
361 The increase of the microbial biomasses due to acclimation was attributed to the fact  
362 that essential oils are a readily decomposable carbon source to microorganisms  
363 (Vokou et al., 2002; Vokou & Margaris, 1988). Moreover, the quality of the  
364 decomposable material explained why there were no significant changes relative to  
365 the control in actinomycetes, which mineralize slowly relatively stable organic carbon  
366 substrates (Sharma, 2014).

367 Acclimation appeared to act selectively on members of the microbial community  
368 potentially favouring G<sup>+</sup> bacteria which are more resistant to the denaturation of the  
369 cellular membranes that was caused by monoterpenes contained within the oil (Cox et  
370 al., 2000). However, apart from bacteria, the repeated application of low doses of *M.*  
371 *spicata* oil into soil also stimulated fungi. This denotes that although most essential  
372 oils exert inhibitory effects on phytopathogenic fungi by inhibiting mycelium growth  
373 (Dewitte, Landschoot, Carrette, Audenaert, & Haesaert, 2019), there are some species

374 of fungi that could be acclimated to low doses of oil and even favoured when  
375 subsequently exposed to elevated oil concentrations.

376 In contrast to the acclimation responses, pre-treatment of samples with AMF  
377 inoculum caused a reduction in the total microbial biomass as well as the biomass of  
378 bacteria ( $G^+$  and  $G^-$ ), fungi and micro-eukaryotes after stress exposure. A possible  
379 explanation could be the competitive interactions among microbes favoured by high  
380 dose oil exposure and those contained in the AMF inoculum (AM fungus and bacteria  
381 attached on fungal spores) or those affected positively by inoculation. Competition  
382 may be present for N since among others, fungal hyphae take up nitrogen in the form  
383 of  $NH_4$  and/or  $NO_3$  to meet their large N requirements (Hodge & Fitter, 2010).

384 It is worth noting that the relative changes in microbial biomasses when the two  
385 pre-treatments (acclimation and inoculation) were combined were similar to the  
386 controls. This potentially indicates opposite effects of the two pre-treatments  
387 (acclimation versus inoculation) but this was not reflected in the activities of  
388 asparaginase, glutaminase and arylamidase. These enzymes were sensitive indicators  
389 of the joint pre-treatment effect on the subsequent response of soil function to stress.  
390 Samples exposed to a combination of both pre-treatments and subsequently stressed  
391 exhibited increased activity of asparaginase and glutaminase and reduced activity of  
392 arylamidase compared to stress only exposed samples. Previously, Stamou et al.  
393 (2017) suggested a synergistic effect of inoculation and acclimation for asparaginase  
394 and glutaminase activity before stress exposure. This effect appears to continue post  
395 stress exposure and is explained by the fact that stress on its own caused no  
396 significant effect on enzyme activities. However, changes in activities would be  
397 expected over time period, when the microbial community selected by a stress would  
398 produce enzymes in order to meet nutrient demands.

399 Acclimation imposed the negative response of urease to stress. This is in  
400 accordance with the findings of Papatheodorou, Margariti, & Vokou (2014); R-  
401 carvone which is the main constitute of *M. spicata* oil had an inhibitory effect on  
402 urease activity even at a low dose. A negative response was recorded for arylamidase,  
403 while this **was not observed** for glutaminase and asparaginase since the application of  
404 low dose oil acted as stimulus. The effect of AMF inoculation on enzymes was in all  
405 cases positive. AMF by altering the root exudation pattern or by producing exudates  
406 from their hyphae (Bharadwaj, 2007) offer carbon for growth and metabolism to  
407 microbes in the rhizosphere. The enhanced provision of carbon could be followed by  
408 an increased availability of nutrients. The latter is achieved by increased enzyme  
409 activity.

410

#### 411 **Relationships** between structure and function

412 In order to identify relationships among features of the microbial community and  
413 soil functions we employed a NIPALS analysis. To the best of our knowledge, even  
414 though soil functions are partially controlled by soil microbes, non-synchronized  
415 changes in the microbial community structure and soil functions have been reported  
416 previously (Bowles, Acosta-Martínez, Calderón, & Jackson, 2014). According to  
417 Graham et al. (2016), when microbial community structure was inserted as a variable  
418 in a model that related the structure of the microbial community to C and N cycling,  
419 the accuracy of the model was **enhanced** by 29%. Strickland, Lauber, Fierer, and  
420 Bradford (2009) found that differences in the composition of the microbial  
421 community **accounted** for 20% of the variation in the total C mineralisation.

422 Non-synchronizing changes were found in the current experiment but before the  
423 initiation of essential oil stress (Stamou et al., 2017). Three days **post stress**, NIPALS

424 analysis showed (Fig.3) that specific enzymes were ordinated together with microbial  
425 ratios in samples inoculated, acclimated and stressed. The relationship between  
426 enzyme activities and certain microbial ratios denotes that specific activities depended  
427 on the relative abundance of specific functional groups (e.g. G<sup>+</sup> or G<sup>-</sup>) rather than on  
428 their biomass *per se*. The activities of asparaginase, NAG and to a lesser extent  
429 glutaminase tended to be related to G<sup>+</sup>/G<sup>-</sup>, Iso/Ant and Sat/Unsat ratios. The positive  
430 relationship between G<sup>+</sup>/G<sup>-</sup> and Sat/Unsat ratios was **unsurprising given** that most G<sup>+</sup>  
431 bacteria contain PLFAs with single bonds (saturated) in their cellular membranes.  
432 (Cho & Salton, 1966). Since asparaginase is produced by both G<sup>-</sup> and G<sup>+</sup> bacteria (like  
433 *Bacillus* sp. (G<sup>+</sup>), *Corynebacterium glutamicum* (G<sup>+</sup>), *Pseudomonas* sp. (G<sup>-</sup>), *Erwinia*  
434 sp. (G<sup>-</sup>) and *Eschericia coli* (G<sup>-</sup>); Asthana and Azmi, 2003), the positive relationship  
435 of the G<sup>+</sup>/G<sup>-</sup> ratio to asparaginase activity is partially interpretable. Our results are in  
436 accordance with Dong et al. (2015) who reported a significant correlation between  
437 NAG activity and the G<sup>+</sup>/G<sup>-</sup> ratio in a nutrient-enrichment experiment. However, they  
438 presented no explanation for this relationship. High values of Iso/Ant and Sat/Unsat  
439 ratios are indicative of carbon or nutrient limitation (Bossio & Scow, 1998; Fierer,  
440 Schimel, & Holden, 2003) occurring in inoculated, acclimated and then stressed  
441 samples. It is likely that the addition of the essential oil either repeatedly (acclimation)  
442 or **as a single stress episode** provided a large C source for microbial growth. In  
443 addition, fungal hyphae absorb N from **the** soil, inducing N limitation to microbes. In  
444 response microbes increased the production of asparaginase, glutaminase and NAG to  
445 meet their demands in N. These findings support the microbial resource allocation  
446 theory according to which microbes expend energy to produce enzymes when  
447 nutrients are short in supply (Stone, Plante, & Casper, 2013). In contrast, such

448 limitation did not occur in acclimated and stressed samples as supported by the  
449 increased biomass of all microbial groups in those samples.

450 Based on our findings, we suggest that in order to predict soil functions it would be  
451 more useful to construct models incorporating variables related to microbial ratios  
452 rather than based on the absolute biomass values.

453

## 454 **Conclusions**

455 In relation to the first hypothesis presented, we confirm that the two microbial soil  
456 communities (pre-selected via acclimation or AMF inoculation) responded differently  
457 to stress exposure. Each treatment affected by its own the response of microbial  
458 groups to stress. Acclimation enhanced the biomass of G<sup>+</sup> bacteria, fungi and micro-  
459 eukaryotes confirming a priming effect of a low intensity stimulus when applied  
460 repeatedly. AMF inoculation decreased the biomass of these microbial groups.  
461 Enzymes appeared sensitive indicators of pre-treatment effects to stress response. The  
462 activity of glutaminase, asparaginase and NAG were affected positively by both pre-  
463 treatments while that of urease was negatively by acclimation. We conclude that  
464 synchronization between microbial ratios and enzymes can be found under conditions  
465 of multiple effects (inoculation, acclimation, stress). However, this needs much  
466 further work to be tested.

467

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472

473 **Conflict of interest**

474 The authors have declared that no conflict of interest exists.

475

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For Review Only



681 Table 1. Results provided by an ANOVA applied to data by Generalized Linear  
 682 Models (GLM). Values of the Wald statistic and p were also given. The Wald statistic  
 683 was tested against the Chi-square distribution (degree of freedom 1)

684

	AMF		Oil		AMF xOil	
	Wald ( $X^2_1$ )	p-value	Wald ( $X^2_1$ )	p-value	Wald ( $X^2_1$ )	p-value
Total	7.31	0.007	11.19	0.0008	-	-
G <sup>+</sup> bacteria	8.42	0.004	13.82	0.0002	-	-
G <sup>-</sup> bacteria	4.37	0.04	-	-	-	-
Actinomycetes	-	-	-	-	-	-
Fungi	3.95	0.047	10.00	0.002	-	-
Micro- eukaryotes	13.27	0.0003	24.04	0.00001	-	-
Arylamidase	15.52	0.00008	8.32	0.004	8.93	0.003
Asparaginase	6.99	0.008	7.09	0.005	13.01	0.0003
Glutaminase	14.24	0.0001	18.58	0.00002	9.99	0.002
NAG	8.89	0.003	-	-	-	-
Phosphatase	-	-	-	-	-	-
Urease			4.31	0.04		

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688 **Figures' Legends**

689 **Figure 1.** Percent changes in the biomasses of the different microbial groups  
690 relative to control, three days after stress exposure in three different treatments; only  
691 inoculated (AMF), only acclimated (AC), jointly inoculated and acclimated samples  
692 (AMF+AC, Joint). The number of asterisks correspond to level of significance (\*:  
693  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ) while their colour correspond to specific effect  
694 (red: AMF, green: AC, blue: AMF+AC)

695

696 **Figure 2.** Percent changes in the activity of different enzymes relative to control,  
697 three days after stress exposure in three different treatments; only inoculated (AMF),  
698 only acclimated (AC), jointly inoculated and acclimated samples (AMF+AC; Joint).  
699 The number of asterisks correspond to level of significance (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ,  
700 \*\*\*:  $p < 0.001$ ) while their colour correspond to specific effect (red: AMF, green: AC,  
701 blue: AMF+AC)

702

703 **Figure 3.** Ordination of treatments (+AMF (inoculated). +AC (acclimated)),  
704 enzyme activities, microbial biomasses and their ratios(underlined letters) at the phase  
705 of the two first components space on a PCA biplot, produced by applying the PLS  
706 algorithm on data.

707

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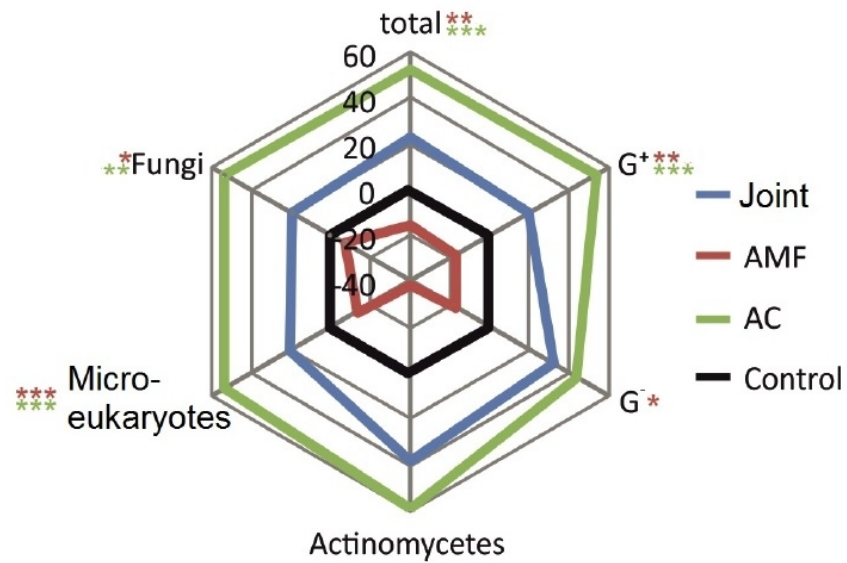


Figure 1. Percent changes in the biomasses of the different microbial groups relative to control, three days after stress exposure in three different treatments; only inoculated (AMF), only acclimated (AC), jointly inoculated and acclimated samples (AMF+AC, Joint). The number of asterisks correspond to level of significance (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ) while their colour correspond to specific effect (red: AMF, green: AC, blue: AMF+AC)

221x132mm (96 x 96 DPI)

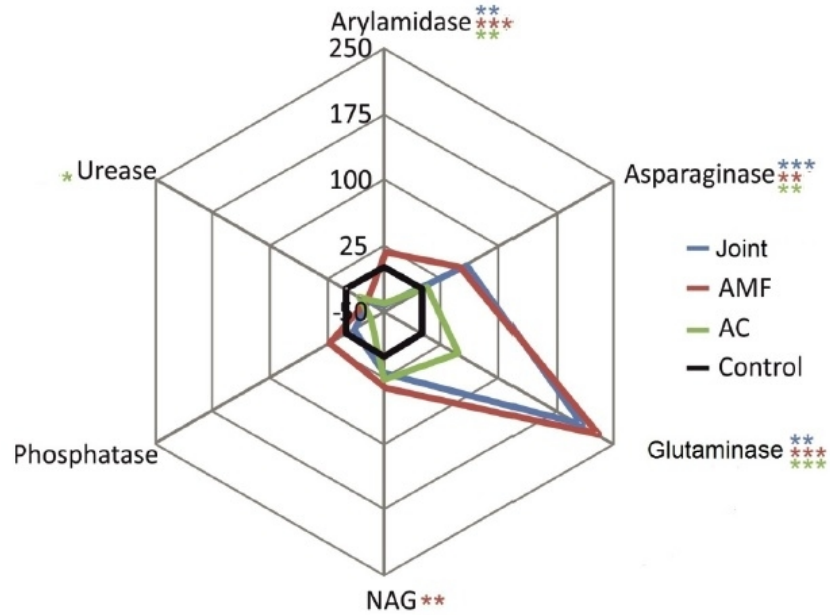


Figure 2. Percent changes in the activity of different enzymes relative to control, three days after stress exposure in three different treatments; only inoculated (AMF), only acclimated (AC), jointly inoculated and acclimated samples (AMF+AC; Joint). The number of asterisks correspond to level of significance (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ) while their colour correspond to specific effect (red: AMF, green: AC, blue: AMF+AC)

195x143mm (96 x 96 DPI)

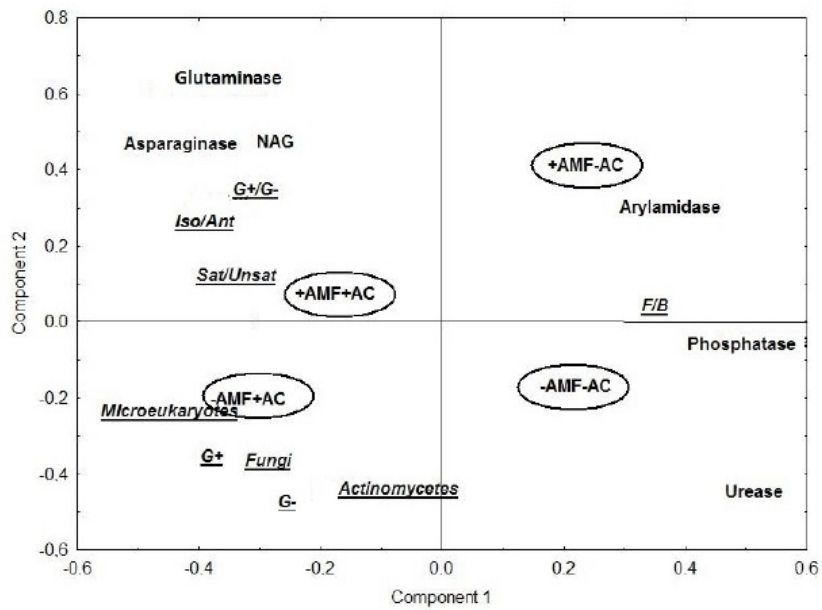


Figure 3. Ordination of treatments (+AMF (inoculated). +AC (acclimated)), enzyme activities, microbial biomasses and their ratios (underlined letters) at the phase of the two first components space on a PCA biplot, produced by applying the PLS algorithm on data.

195x134mm (96 x 96 DPI)