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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 (*Mentha spicata* 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) *Rhizophagous irregularis.* 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, 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*.

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

Introduction

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

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

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

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

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

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

Materials and Methods

Experimental Design

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

of organic C and nutrients before sterilization was C 16200 mg/Kg, N 960 mg/Kg, Pextr 21 mg/Kg, K 1.62 mg/Kg, Ca 1.17 mg/Kg, Mg 0.18 mg/Kg and Na 0.136 mg/Kg. This chemical composition ensured the successful colonization of plants with AMF. To eradicate indigenous AMF and other soil borne biota, the soil-sand mixture was sterilized by autoclaving (4 h at 120 ^{0}C).

For half of all pots (n=9), the roots of tomato seedlings were inoculated with 10 g of *Rhizophagous irregularis* inoculum whilst the other half (n=9) were non-inoculated. The inoculum consisting of spores and hyphal fragments of *R. irregularis,* provided by the Energy and Resource Institute, India at a concentration of1000 propagules per gram of inoculum clay powder. Prior to the experiment, to test the quality of the inoculum,it was applied in 5 mixed pot cultures of *Plantango lanceolata*, *Dactylis glomerata*, and *Trifolium repens,* and the colonization rate was estimated 40, 60 and 80 days after the application of the inoculum. In all cases the root colonization rate was >80%, 40 days after the application.

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

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

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

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

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

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

Soil sampling

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

AMF analysis

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

Enzymatic activity assays

The activities of six soil enzymes were studied. These were N-acetyl-glucosaminidase (NAG), acid phosphatase, urease, asparaginase, glutaminase and arylamidase. N-acetyl-glucosaminidase (NAG) and acid phosphatase activities were determined according to the procedures of Allison and Jastrow (2006), as these were modified in order to be applicable for 96-well microplates. Approximately 1-2 g fresh soil (equivalent to 0.5 g dry weight) were added in 60 ml of 50 mM sodium acetate buffer, pH 5, and homogenized in a blender for 1min. Then, 50 μL of homogenized soil slurry were combined with 150 μL substrate solution and incubated for 3 h (NAG) or 1 h (acid phosphatase) at 21 ^{o}C under constant shaking. Substrate solutions were 2 mM p-nitrophenyl-*β*-N-acetylglucosaminide for NAG and 5 mM p-nitrophenyl-phosphate for phosphatase, all in acetate buffer. After incubation, 100 mL of the slurry-substrate supernatant (without soil particles) were carefully transferred to another microplate for colorimetric determination of product concentrations. The *p*-nitrophenol (pNP) reaction product from the phosphatase and NAG assays was measured at 405 nm, after addition of sodium hydroxide. Eight replicates were run per sample; in each case, we included appropriate controls to estimate the background absorbance of the substrate and homogenate. The activity of the two enzymes is presented in units of μmol pNP g^{-1} dry soil h^{-1}.

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

Urease activity was measured spectrophotometrically at 610 nm. Activity is expressed as micromoles of ammonium released per hour per g soil (μmol NH_4^+ g^{-1} h^{-1}).

The activities of asparaginase and glutaminase were determined according to the methods of Tabatabai (1994). Briefly, the methods were based on the determination of NH_4^+ released when soil is incubated at 37 °C for 2 h with 0.1 M tris-hydroxymethyl-aminomethane (THAM) buffer, toluene and L-asparagine or L-glutamine for asparaginase and glutaminase, respectively. The NH_4^+ released was determined by treating the incubated soil sample with 2 M KCl containing Ag_2SO_4 (to stop the enzymatic activity) followed by steam distillation of an aliquot of the resulting soil suspension with MgO. The activities of these enzymes were assayed on <2 mm field-moist samples, at the optimal pH value, in duplicates and one control, and are expressed on a moisture-free basis. Moisture was determined after drying at 105 °C for 24 h.

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

Phospholipid fatty acid analysis

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

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

The total amount of PLFAs was used to account for the total microbial biomass. Overall, 22fatty acid methyl esters were identified and considered for further analysis, including the internal standard 19:0. These are i-15:0, a15:0, 15:0, i16:0, i17:0 which were indicators of $Gram^+$ bacteria (McKinley, Peacock, & White, 2005; Myers, Zak, White, & Peacock, 2001; Zak et al., 1996), the bacteria indicators 16:0, 17:0 (Rillig, Mummey, Ramsey, Klironomos, & Gannon, 2006), the $Gram^-$ bacteria indicator 16:1ω9c (Zak et al., 1996) and the indicators of actinomycetes 10Me16:0, 10Me17:0, 10Me18:0 (Frostegård, Tunlid, & Bååth, 1993; White, Stair, & Ringelberg, 1996). All these were considered to be of bacterial origin only and were chosen to represent bacterial biomass. The 18:1ω9c and 18:2ω9,12 fatty acids were used as indicators of fungal biomass (Zak et al., 1996; Rillig et al., 2006) and the fatty acid 16:1ω5 was used as indicator of AM fungi and specifically of viable hyphal biomass because the fungal storage reserves such as spores, vesicles and propagules are represented by neutral 16:1ω5 (Olsson & Johansen, 2000). The PLFAs 20:0, 22:0, 23:0, 24:0 were considered as indicators of microeukaryotes (algae, protozoa, nematodes; (Smith et al., 1986). Finally the fatty acids 17:1, 18:0 and 14:0 were mainly of microbial origin. Moreover, the ratios $Gram^+/Gram^-$ (G^+/G^-), fungi/bacteria (F/B), iso/anteiso (Iso/Ant) and Saturated/Unsaturated (Sat/Unsat) were estimated. Iso biomass is equal to the

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

Data analyses

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

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

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

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

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

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

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

Results

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

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

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

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

Discussion

Microbial community structure and enzyme activity

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

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

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

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

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

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

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

Relationships between structure and function

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

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

analysis showed (Fig.3) that specific enzymes were ordinated together with microbial ratios in samples inoculated, acclimated and stressed. 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*. The activities of asparaginase, NAG and to a lesser extent glutaminase tended to be related to G^+/G^-, Iso/Ant and Sat/Unsat ratios. The positive relationship between G^+/G^- and Sat/Unsat ratios was unsurprising given that most G^+ bacteria contain PLFAs with single bonds (saturated) in their cellular membranes. (Cho & Salton, 1966). Since asparaginase is produced by both G^- and G^+ bacteria (like *Bacillus* sp. (G^+), *Corynebacterium glutamicum* (G^+), *Pseudomonas* sp. (G^-), *Erwinia* sp. (G^-) and *Eschericia coli* (G^-); Asthana and Azmi, 2003), the positive relationship of the G^+/G^- ratio to asparaginase activity is partially interpretable. Our results are in accordance with Dong et al. (2015) who reported a significant correlation between NAG activity and the G^+/G^- ratio in a nutrient-enrichment experiment. However, they presented no explanation for this relationship. High values of Iso/Ant and Sat/Unsat ratios are indicative of carbon or nutrient limitation (Bossio & Scow, 1998; Fierer, Schimel, & Holden, 2003) occurring in inoculated, acclimated and then stressed samples. It is likely that the addition of the essential oil either repeatedly (acclimation) or as a single stress episode provided a large C source for microbial growth. In addition, fungal hyphae absorb N from the soil, inducing N limitation to microbes. In response microbes increased the production of asparaginase, glutaminase and NAG to meet their demands in N. These findings support the microbial resource allocation theory according to which microbes expend energy to produce enzymes when nutrients are short in supply (Stone, Plante, & Casper, 2013). In contrast, such

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

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

Conclusions

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

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Conflict of interest

The authors have declared that no conflict of interest exists.

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679
680

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

	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^+bacteria	8.42	0.004	13.82	0.0002	-	-
G^-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		

687

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

708

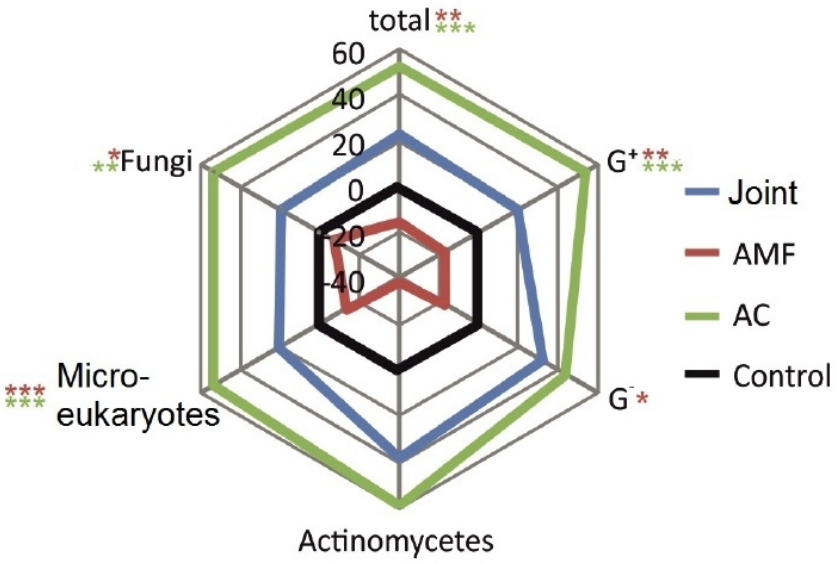

Figure 1. Percent changes in the biomasses of the different microbial groups relative to control, three days after stress exposure in three different treatments; only inoculated (AMF), only acclimated (AC), jointly inoculated and acclimated samples (AMF+AC, Joint). The number of asterisks correspond 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)

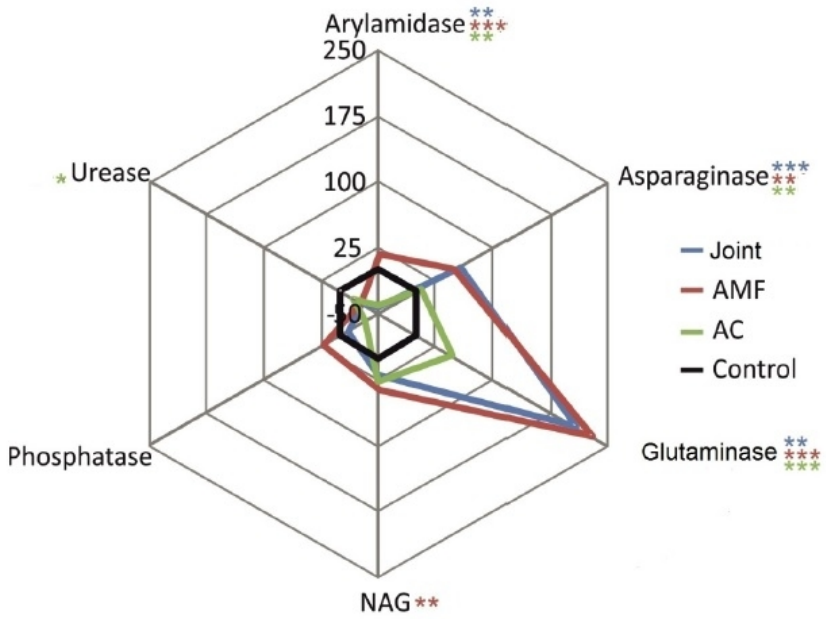

Figure 2. Percent changes in the activity of different enzymes relative to control, three days after stress exposure in three different treatments; only inoculated (AMF), only acclimated (AC), jointly inoculated and acclimated samples (AMF+AC; Joint). The number of asterisks correspond to level of significance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

195x143mm (96 x 96 DPI)

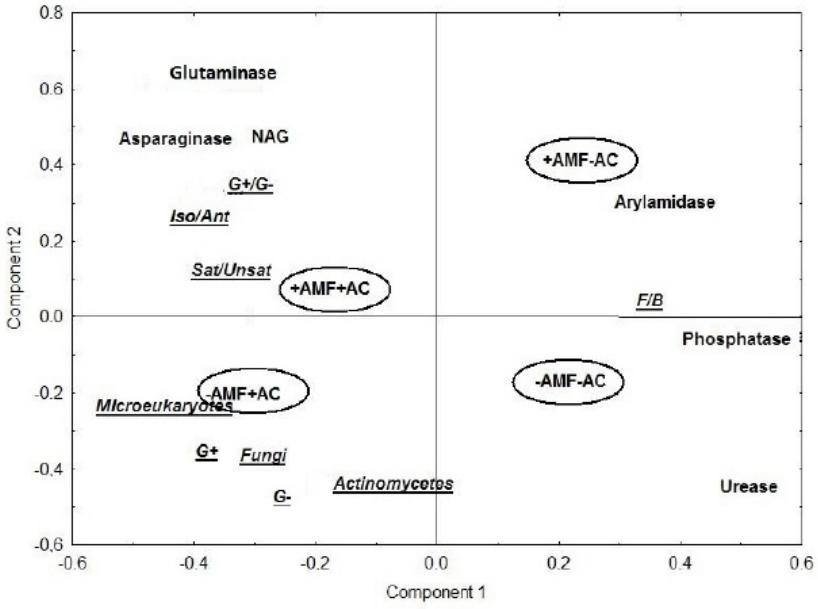

Figure 3. Ordination of treatments (+AMF (inoculated). +AC (acclimated)), enzyme activities, microbial biomasses and their ratios (underlined letters) at the phase of the two first components space on a PCA biplot, produced by applying the PLS algorithm on data.

195x134mm (96 x 96 DPI)