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Title: Short-term plant legacy alters the resistance and resilience of soil microbial communities exposed to heat disturbance in a Mediterranean calcareous soil.

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Abstract

Plant legacy is a concept representing the effects exerted by plants on soil once they are no longer growing. We hypothesized that plant species and mixture (intercropping) would induce different short-term legacy effects impacting carbon and nitrogen-related soil microbial activities and resistance and resilience after a heat disturbance. A microcosm experiment was conducted using a calcareous Mediterranean soil conditioned by a complete vegetative cycle in a greenhouse with four planting modalities (W = monoculture of Wheat (*Triticum aestivum* L.); L = monoculture of white Lupin (*Lupinus albus* L.); WL = both species intercropped; U = unplanted soil). Half of microcosms were incubated at 28°C (C = control conditions) whereas the remaining half were exposed at 48°C for 2 days (S = stress conditions), with an immediately return to control conditions. Microcosms were destructively sampled at 2, 7, 16 and 28 days (T2, T7, T16, T28) after the end of the heat disturbance and the following soil measurements were performed: Basal Respiration (BR), Substrate-Induced Respiration (SIR), Nitrification Enzyme Activity (NEA) and N mineral concentrations. Our results demonstrated that monocultures and intercropping promoted different legacy effects under control conditions especially for SIR. WL soils presented lower values of SIR than L and higher than W soils. For SIR, W and WL soils conferred greater resistance to the heat stress, whereas L and WL soils conferred higher resilience at T28. For NEA, no differences between soils were observed for resistance to heat stress, but at T16, soils having WL legacy were more resilient than L soils, but comparable to those having W legacy. Our results highlight that a short-term legacy effect is measurable but greatly differs between C- and N-related microbial activities. We estimated that intercropping had modified ability of soil microorganisms to face heat stress, suggesting that plant legacy effect has to be considered to mitigate extreme climatic events in Mediterranean soils.

48 **Keywords:** climate change; soil respiration; nitrification; crop mixture; plant soil feedback,
49 heat stress.

1. Introduction

Substantial legacy effects on soil properties are induced by plants and can persist after the disappearance of those plants that were responsible for these effects (van Der Putten et al., 2013). Plant legacy effects have been pointed out to influence carbon storage in soils (Lange et al., 2015), succession in plant communities (van der Putten et al., 2016) or plant invasions (Bailey and Schweitzer, 2016). Legacy effects of plants on soils are largely driven by shifts in soil microbial communities. Indeed, plant roots can directly alter soil microbial communities by releasing carbon compounds named rhizodeposits (Eisenhauer et al., 2017; Hunter et al., 2014; Kuzyakov and Blagodatskaya, 2015). Rhizodeposits include a wide range of organic compounds including specific root exudates that shape microbial communities in root vicinity, *i.e.* the rhizosphere microbiome (Dennis et al., 2010; Li et al., 2018). Furthermore, composition of rhizodeposits is known to be plant specific (Hunter et al., 2014; Li et al., 2018), which suggests that a single species will affect differently soil microbial communities compared to a plant mixture (Chen et al., 2019; Eisenhauer et al., 2017; Tang et al., 2014; Tang et al., 2016). Plants are also able to alter soil microbial communities indirectly by modifying the abiotic properties of the soil, *i.e.* pH, nutrient availability, moisture or soil structure (Hinsinger et al., 2009). Several plant legacies were characterized in grasslands (Grman and Suding, 2010; Lange et al., 2015; Strecker et al., 2016; Zak et al., 2003), but debated in agroecosystems while recent publication pointed out its implication in shaping soil microbiome (Li et al., 2018). In agroecosystems, the frequency of perturbations due to annual practices and the low plant diversity barely support comparison to natural ecosystems. However, some agronomic practices such as intercropping are in rupture with conventional agriculture, as it consists to grow two or more species or genotypes in the same field during a significant period of their life cycle (Brooker et al., 2015). Intercropping is of great interest in many countries as a solution to replace synthetic inputs by ecological processes (Brooker et

al., 2015; Doré et al., 2011; Malézieux et al., 2009). Long-term legacy effects of intercropping have been estimated by Cong et al. (2015), but within 1-year timespan authors are reluctant to expect a significant effect on soil communities. However, Pivato et al. (2017) estimated that after only 12 weeks of bi-species culture, N cycling-related microbial communities were strongly affected especially at low N level compared to each species in monoculture. Grman and Suding (2010) also estimated a short-term legacy effect pointing out the multiple drivers defining both plant legacy duration and intensity. Given the crucial role of soil microbial activities in ecosystem functioning, these plant legacy effects can contribute to agroecosystem stability in the context of climate change and particularly during extreme events. Several studies have been focused on resistance (capacity to withstand change) and resilience (capacity for recovering of function) of C-related soil microbial activities to environmental disturbances (Bérard et al., 2012; Bérard et al., 2011; Chaer et al., 2009; Guillot et al., 2019; Hamdi et al., 2011; Mooshammer et al., 2017; Orwin and Wardle, 2005). However, studies about stability of more specialized soil microbial communities like nitrifiers are scarce (Mooshammer et al., 2017; Thion and Prosser, 2014; Wertz et al., 2007). Yet there are reasons to argue that plants are able to shape soil related N-cycle communities (Zak et al., 2003). Our rationale is to consider that diverse plant species yield different legacies, ultimately contributing to alleviate effects of extreme climatic events as heat waves in agroecosystems. We hypothesized that intercropped species promote a (i) different legacy effect on soil microbial processes in comparison to single species, and accordingly (ii) influence the resistance and resilience of soils. We focused on nitrification and respiration microbial processes (potential nitrification, substrate-induced respiration and basal respiration) as key processes of the N and C cycles in environmental and plant productivity issues. To achieve this goal, we generated a novel plant legacy in a calcareous cambisol, through a greenhouse experiment conducted with a soil collected in a Mediterranean site near

Montpellier, France. Soil was collected 3 months after a pea crop, sieved at 1-cm to be homogenized and ridded of any coarse organic material, then air dried and stored at ambient temperature before use. In 2017, soil was mixed with perlite and conditioned by one complete vegetative cycle of two crop species grown as single crops (monocultures), (i) white lupin (*Lupinus albus* L.) and (ii) bread wheat (*Triticum aestivum* L.), as well as the mixture of both species (intercrop). Finally, we calculated the resistance and resilience indices (*sensu* Orwin and Wardle (2004)) after a heat disturbance.

2. Material and methods

2.1. Soil origin and greenhouse experiment before heat stress experiment

Soil originated from a plot of the INRA experimental station localized at Mauguio (3°59'6"E, 43°37'13"N, 12 m altitude) in French littoral Mediterranean region. The soil was a calcareous cambisol with a clay loam texture according to USDA classification (clay 294 g kg⁻¹, fine silt 200 g kg⁻¹, coarse silt 233 g kg⁻¹, fine sand 156 g kg⁻¹ and coarse sand 118 g kg⁻¹), which had developed on an alluvial calcareous parent material, typical of the South East plains of Montpellier. The regional climate is typically Mediterranean with surface regularly exposed to drying-rewetting cycles due to irregular rain events mainly concentrated in Autumn and Spring. The annual average rainfall at this site during the growing season, from September to June, over the last 20 years was 598 mm. Recorded average soil temperatures at 10 cm depth since 2012 demonstrated that plot had never experienced temperatures higher than 38.4 °C. During the four months before soil sampling, 3 cycles of drought were recorded (*i.e.* 20 days with cumulative rainfall lower than 2mm, according to Tang et al. (2014)), while maximal air temperatures ranged from 22.8 °C (in September) to 34.7 °C (in August). The last precipitation episode (4.8 mm) preceding soil sampling occurred 5 days earlier. Soil was collected (0-15 cm depth) 3 months after pea harvest, air dried and sieved at 1-cm to

homogenize and remove coarse organic material, then finally stored at ambient temperature before use to preserve inherent soil quality (Salomé et al., 2014).

A pot experiment was then conducted in a greenhouse (Centre Mondial de l'Innovation – Roullier Group – Saint-Malo, France) from September to December 2017 with the soil previously described. Before being conditioned in the pots (4-dm³), the soil was re-sieved with a 4-mm mesh to remove any organic material and mixed with perlite to allow better conditions for plant root development. Pots were filled with substrate composed of 2 kg of soil and 0.2 kg of perlite. The characteristics of the substrate were clay 202 g kg⁻¹, silt 454 g kg⁻¹, sand 343 g kg⁻¹, total CaCO₃ 39 g kg⁻¹, pH_w 8.3, CEC_{Metson} 15 cmol₊ kg⁻¹, organic C 10.4 g kg⁻¹, total N 0.95 g kg⁻¹, ammonium (N-NH₄⁺) 20 mg kg⁻¹, nitrate (N-NO₃⁻) 39 mg kg⁻¹. The WHC of the substrate corresponded to 28% of gravimetric humidity. For simplicity, substrate will be named herein as soil. The planting modalities of this experiment were: (i) monoculture of wheat (W) (*Triticum aestivum* L. cv. Lennox) at a density of 4 plants per pot, (ii) monoculture of white lupin (L) (*Lupinus albus* L. cv. Feodora) at a density of 2 plants per pot, (iii) intercropping of both species (WL) at half of the density for each corresponding monoculture (2 wheat and one lupin plants), and (iv) an unplanted soil (U). Each planted and unplanted treatments was replicated 4 times. At maturity, plants (shoots and roots) were harvested and the soil was carefully separated from the roots by gently shaking followed by hand sorting and then kept at 4°C in the dark until microcosm experiment (16 weeks).

2.2. Heat stress microcosm experiment

A total of 128 microcosms were prepared (4 replicates x 4 planting treatments x 2 temperatures x 4 sampling dates) containing 50 g of equivalent dry soil from greenhouse experiment, and placed in 1-dm³ glass jars hermetically sealed by a rubber gasket. Soils of microcosms were rehydrated at 100% of the water holding capacity and pre incubated at 12°C

for 5 days in order to ensure uniform initial experimental conditions. Afterwards, half of the microcosms (64 samples) were incubated at 28°C and the remaining half at 48°C, corresponding to control (C) and heat stress (S) conditions, respectively. All microcosms were placed into darkness in laboratory incubators (KBWF 720, Binder, Germany) with a precision of 0.4 °C. In order to i) avoid any drying-rewetting cycles during the experiment altering the C and N cycles including nitrification (Fierer and Schimel, 2002) and ii) ensure precise estimation of nitrification potential assays conducted with soil slurries (see section 2.3), we kept soil moisture at 100% WHC. To ensure no variations of water regime in microcosms, a 20-cm³ polypropylene container filled with water was placed in each sealed microcosm. Microcosms were regularly weighted to check any soil moisture changes. After two days of heat disturbance at 48°C, the stressed microcosms were transferred back at 28°C. Each microcosm contained an 1M NaOH trap for basal respiration determination (see section 2.3). At each sampling date (T2, T7, T16 and T28 corresponding to 2, 7, 16 and 28 days after the end of the heat stress), samples were immediately split into 4 subsamples for all subsequent soil analyses (substrate-induced respiration (SIR), nitrification enzyme activity (NEA), soil NH₄⁺ and NO₃⁻ measurements, and soil water content). Measurements of SIR and NEA were performed on fresh soils within 2 days after sampling, whereas soil samples for mineral N measurements were frozen (-20°C) until analysis.

2.3. Soil microbial and chemical analyses

Basal respiration (BR) traps were collected and replaced at each sampling date. An aliquot of 1-cm³ of NaOH was back-titrated with 0.1 M HCl, after adding 2.5-cm³ of BaCl₂ solution (30 %) to precipitate the Na₂CO₃ issued from soil CO₂ respiration. Results of BR were expressed in µg C-CO₂ day⁻¹ g⁻¹ of dry soil. SIR measurements were performed on 10 g of equivalent dry soil according to Patra et al. (2005), in presence of glucose (1.2 mg C-

glucose g⁻¹ dry soil). A CO₂ accumulation kinetic was measured using a gas chromatograph (µGC R3000, SRA instruments, France) at 28°C. SIR was expressed in µg C-CO₂ h⁻¹ g⁻¹ of dry soil. NEA was measured with 3 g of equivalent dry soil according to Patra et al. (2005). Briefly, a (NH₄)₂SO₄ solution (50 µg N-NH₄⁺ g⁻¹ dry soil) was added to each soil sample. Samples were placed at 28°C, shaken at 140 rpm to ensure aerobic conditions during 10 hours, and NO₂⁻ + NO₃⁻ production was measured every two hours using a photometer (Smartchem 200, AMS Alliance, France). NEA was expressed in µg of N-(NO₂⁻+NO₃⁻) h⁻¹ g⁻¹ of dry soil. Finally, soil ammonium (N-NH₄⁺) and soil nitrate (N-NO₃⁻) were extracted from a soil:solution ratio of 1:2 with 1M KCl solution after a 30 min of contact at 140 rpm, followed by 30 min of rest. Supernatant were filtered at 0.45 µm and mineral N concentrations were estimated with an Analytical Discrete Multi-Chemistry Analyzer (AQ2, SEAL Analytical, UK). Soil mineral N content were expressed as mg N-NH₄⁺ and mg N-NO₃⁻ kg⁻¹ of dry soil.

2.4. Percentage change relative to control

In order to investigate recovery rate, percentage change of values in stressed samples relative to those in the controls, at each sampling date, were calculated as follows (Bérard et al., 2011; Chaer et al., 2009):

$$relative\ change\ (\%) = \left[\left(\frac{S_i}{C_i} \right) - 1 \right] \times 100 \quad (1)$$

where S_i and C_i are the variable values in the stressed and control soil samples, respectively, at sampling date i (*i.e.* T2, T7, T16 or T28).

2.5. Soil microbial resistance and resilience indices

To investigate only potential differences among soils with specific plant legacy (W, L, WL), unplanted (U) soil was not considered for calculation of resistance and resilience indices (see section 3.3). Resistance (RS) and resilience (RL) of soil microbial activities to a heat stress were assessed by the following indices (Orwin and Wardle, 2004):

$$RS = 1 - \frac{2 \times |D_2|}{C_2 + |D_2|} \quad (2)$$

where D_2 is the difference between the values of the response variable (BR, SIR or NEA) in control (C_2) and stressed conditions (S_2), 2 days after the end of the disturbance;

$$RL = \frac{2 \times |D_2|}{|D_2| + |D_x|} - 1 \quad (3)$$

where D_x is the difference between the control and disturbed soil on 7, 16 or 28 days after the end of the disturbance.

Both indices are bounded by -1 and $+1$, where an index of 1 indicates full resistance (no effect of the disturbance on the response variable) or full resilience (same values of the response variable in control and stressed conditions). For resistance, an index value of 0 indicates either 100% decrease or increase in the response variable compared to control, while a negative index represents a modification greater than 100% in the response variable compared to the control (*i.e.* the soil has low resistance). In regard to resilience, an index of 0 indicates that the disturbed soil has either not recovered after the end of the disturbance (*i.e.* $D_x = D_2$) or that the stressed soil is different to the control by the same amount, but in the opposite direction (*i.e.* $D_x = -D_2$). A resilience index between 0 and 1 indicates that the

response variable has not fully recovered, whereas a negative index value indicates that the absolute value of D_x is higher than the absolute value of D_2 (*i.e.* the soil has low resilience).

2.6. Statistical analyses

The effects of planting legacy (*i.e.* both planted (W, L, WL) and unplanted (U) modalities) and heat stress on all soil variables were tested for each sampling date by two-way ANOVA (planting legacy x heat stress). Afterwards, one-way ANOVA was performed in absence of interaction to test differences among treatments. The effect of specific plant legacy (*i.e.* only planted modalities – W, L, WL) on resistance and resilience indices, as well as the effect of planting legacy on percentages of change in microbial activities relative to controls were also tested by one-way ANOVA, within each sampling date. Significant differences between means were tested by Tukey's multiple comparison tests ($p < 0.05$). Normality of residuals and homogeneity of variance were tested by the Shapiro and Levene's tests, respectively. When necessary, data for microbial activities were squared root-transformed. Principal component analysis (PCA) were performed with all chemical and microbial soil variables. Within a PCA, coordinates of the individuals on each axis were used for testing differences between groups (*i.e.* levels of a factor) by one-way ANOVA or Kruskal-Wallis tests in case of non-normality. Tukey's multiple comparison tests ($p < 0.05$) or Wilcoxon rank sum tests ($p < 0.05$) were then performed accordingly. Finally, a redundancy analysis (RDA) was performed to assess the effect of all soil microbial and chemical variables (SIR, NEA, BR, $N-NO_3^-$ and $N-NH_4^+$ contents) on resistance (at T2) and resilience (at T16) indices. Statistical analyses were performed with R software v. 3.5.1 (R Core Team, 2018). Packages used for the ANOVA, Tukey's test, PCA and RDA analysis were: *car*, *agricolae*, *FactoMineR* and *vegan*, respectively.

3. Results

3.1. Contrasted effects of planting and heat stress on microbial activities and soil mineral N content

The first two axes of the PCA performed on full data set (BR, SIR, NEA, N-NH₄⁺, N-NO₃⁻ measurements for all sampling dates and stress conditions) explained 74.2% of the total variance (Fig. 1). BR, N-NH₄⁺, NEA and N-NO₃⁻ are the major variables contributing to the first axis while SIR was strongly and negatively correlated to the second axis (Table A.1). Both planting legacy (W, L, WL, U) and sampling dates combined with control (C) and stress (S) conditions had a significant impact on values of samples (Kruskal-Wallis $p < 0.001$; Table A.2 and A.3). Along axes 1 and 2, unplanted soil was significantly different from those with W, L and WL legacies (Wilcoxon test, $p < 0.001$), with centroids showing negative and positive values, respectively (Fig. 1a). Considering sampling dates combined with control and stress conditions (Fig. 1b), earlier dates were significantly distinct between control and heat stress. However, at these earlier dates, the unplanted stressed soils were closer to their controls, suggesting a greater resistance to heat stress. Interestingly, during the period of resilience, the trajectories of the control and stressed soils evolved along the first axis, and at the later dates (T16, T28) stressed and control soils were not significantly different from each other. At all dates combined, a clear difference was observed between stress and control conditions along the second axis (Wilcoxon test, $p < 0.001$), with all centroids of the stressed samples showing positive values, whereas centroids of controls showed negative values.

3.2. Legacy effect of planting on soil C- and N- related activities in response to heat stress

Planting legacy was significant for BR values at the first two dates of measure ($p < 0.001$; Table 1 and Fig C.1). At these dates, L soils clearly differed from unplanted soils, with L soils showing values 1.9-fold greater, when combining control and stress conditions. The planting legacy was still significant at T16, but no effect was observed at T28. An important effect of heat stress was also observed on BR values, particularly at T2 and T7 ($p < 0.01$ and $p < 0.001$, respectively). At the two first dates, stressed soils presented values 1.35-fold greater than control soils on average. At T16 the difference between stressed and control soils was lower, whereas no significant difference between stressed and control soils was observed at T28.

In regard to SIR, planting legacy ($p < 0.001$), heat stress ($p < 0.001$) and planting x stress interaction ($p < 0.01$ at T28 and $p < 0.001$ at other dates) had a strong effect on SIR values (Table 1 and Fig. C.2). Significant differences between planting legacies of control soils were observed at all sampling dates. However, heat stress alleviated these differences. For instance, at T2 (control), SIR means ranked as follows: $L > WL > W = U$, whereas no differences were noticed after stress. One should note that L soils exhibited the greatest SIR values, while unplanted soils presented the lowest. Conversely to BR, stressed conditions exhibited a dramatic decrease of SIR activities compared to the controls, whatever the sampling date. The most important difference was observed at the first date, when stressed soils were 3.6-fold lower than controls. The L soils were particularly altered at the first date because SIR activity were 5-fold lower than controls. This difference between stress and control conditions was also present at the other dates, but decreased with time.

A very strong effect of planting legacy on NEA values was observed, whatever the sampling date ($p < 0.001$; Table 1 and Fig. C.3). Unplanted soils exhibited the greatest values compared to planted soils (2.2- to 3.8-fold greater than W, L and WL). The heat stress effect on NEA was particularly observed at T2 and T7 ($p < 0.001$). At these dates, a significant

decrease in NEA values was observed between controls and stressed soils, but to a lesser extent than that observed for SIR. At T16, heat stress had no effect on NEA, whereas at T28, a significant effect of heat stress ($p < 0.05$) and interaction of heat stress and planting legacy ($p < 0.001$) were observed. An interesting dynamic was observed from T16 to T28, as NEA values increased particularly for stressed (S) soils, and to a lesser extent for control (C) soils. Indeed, at T28, there was a final ranking with L values becoming closer to U values ($U_C > U_S = L_S \geq L_C = W_S = WL_S > WL_C = W_C$).

3.3. Resistance and resilience of soil microbial activities

Recovery rates are illustrated in Fig. 2. Just after heat disturbance (T2), SIR activity was severely altered (-70%, on average) compared to NEA (-46%) and BR (+36%). The most important decrease at T2 was observed for L soils, with a reduction of 79% in SIR relative to control samples. At this date, L soil also exhibited the largest increase in BR (+59%) relative to control samples. Conversely, for NEA, T2 showed the lowest decrease for the unplanted soil (-15%). Focusing on the other dates for SIR, significant differences between percentages of change were only observed at T7, as follows: $U > W = WL > L$ (Table B.1). For NEA in T16, percentages of change for WL and L, but not for W soils, switched from negative to positive. Significant differences between values were observed for NEA at T28. The W soils exhibited greater NEA values in stressed soils relative to controls and compared to WL and L (Table B.1).

Indices of resistance (RS) and resilience (RL) are presented in Table 2. Significant differences in resistance between soils were only observed for SIR ($p < 0.05$). The W soils exhibited the highest RS index, which was significantly different from L, but not from WL. For resilience indices, significant differences between soils for NEA at T16 ($p < 0.01$) and for SIR at T28 ($p < 0.05$) were observed. The RL indices for NEA of both WL and W soils were

greater than that of L soils. The RL indices of L soils were negative at T7, T16, and T28, meaning a very low resilience of NEA. For SIR at T28, the highest RL indices were found for L and WL soils.

The ordination plots of RDA performed on resistance (RS), at T2, and resilience (RL), only at T16, indices clearly discriminated the unplanted soil (Fig. 3 a,b). The resistance indices of NEA were positively associated to NEA and N-NO_3^- concentrations in soils, and negatively to N-NH_4^+ contents. The RS indices of SIR were negatively associated to BR. Considering resilience indices of NEA, these latter were negatively linked to NEA and N-NO_3^- , whereas BR resilience indices were negatively linked to SIR and RL of SIR. Finally, considering soils with only specific plant legacies (W, L, WL), L soils slightly distinguished from the other soils on RDA plots for resistance, and strongly differed from the other soils for resilience. Thus, these results confirmed percentages of change (Fig. 2) and RS and RL indices (Table 2). The L soils were clearly different, being characterized by lower RS of SIR and RL of NEA (Fig. 3 a,b).

4. Discussion

4.1. Legacy effect of plants on soil microbial activities under control conditions

Marked differences were observed between unplanted and planted soils, suggesting that plants were responsible for a legacy effect. Unplanted soils always exhibited greater values of NEA than those for W, L and WL. As plants are able to shape N-related microbial communities (Moreau et al., 2015; Pivato et al., 2017), it occurred that all planted treatments had a negative legacy effect on the activity of nitrifiers. This negative legacy effect may be attributed to the competition between plants and microorganisms for N (Cantarel et al., 2015; Kuzyakov and Xu, 2013; Xu et al., 2011). According to Schimel and Bennett (2004) plants are effective competitors for ammonium which is also the substrate for nitrifiers, reducing

their abundance and activity. Some plant morphological and/or physiological characteristics can also affect the diversity of nitrifiers (Cantarel et al., 2015; Legay et al., 2014; Pommier et al., 2018), or inhibit nitrification (Subbarao et al., 2012). According to these recent results, one should hypothesize that nitrifiers community is shaped by the environment (Erguder et al., 2009; Prosser and Nicol, 2012; Zhalnina et al., 2012), or by plant N-use strategy (Thion et al., 2016). Thereby, we can reasonably suggest a plant legacy effect on nitrification. A great contrast between unplanted and planted soils was also observed for BR and SIR values, with lower values of U compared to W, L and WL. This is consistent with Orwin and Wardle (2005), who observed lower values of soil BR and SIR for bare soil compared to the planted grass treatments. As large amounts of C-compounds can be released by roots as rhizodeposits, e.g. sugars, organic acids, amino acids, mucilage, root or border cells (Kuzyakov and Blagodatskaya, 2015; Philippot et al., 2013), an increase in microbial biomass and activities (Tang et al., 2014) is expected in planted compared to unplanted soils.

Considering only planted soils, we also observed different legacy effects among the modalities (W, L, WL), which is consistent with our first hypothesis. Indeed, except for the first sampling date, greater values of NEA were observed for L soil, compared to W and WL, *i.e.* a stronger legacy effect for lupin than wheat. Le Roux et al. (2013) also reported a positive relationship between the presence of legumes and NEA, which was explained by the build-up of nitrifying communities in response to greater N-NH_4^+ availability in soils, probably due to the release of N derived from Rhizodeposition (NdfR) by legumes (Duchene et al., 2017). In addition, legumes are usually considered less competitive for soil mineral N compared to cereals, which could suggest a higher availability of mineral N in soils under legumes (Mallarino and Wedin, 1990). L soils were also remarkable regarding to C-related microbial variables, showing the greatest values of SIR whatever the date of measurement, as well as the highest values of BR at T2 and T7. White lupin is well known to exude large

quantities of organic anions, like citrate (Dissanayaka et al., 2015; Veneklaas et al., 2003; Wang et al., 2010), compared to cereals and other legumes (Wang et al., 2016). Such exudates may have promoted SIR and BR in our study. This positive legacy effect of white lupin on C-related microbial variables compared to intercropped wheat and white lupin is however not in agreement with the commonly observed positive effect of plant diversity on soil microbial biomass and respiration (Chen et al., 2019; Eisenhauer et al., 2017; Tang et al., 2014). Accordingly, one should note that legume legacies may be further investigated in agroecosystems in order to decipher their potential role in rotation or in intercropping.

4.2. Effects of experimental conditions on C-related microbial activities: Basal Respiration and Substrate-Induced Respiration

Amongst response variables, SIR was the most affected by heat stress (T2). Samples affected by stress presented reductions varying from 65% to 79% relative to controls. As SIR may be considered as a proxy of the active microbial biomass (Anderson and Domsch, 1978; Orwin and Wardle, 2005), these results suggest a dramatic decrease in microbial biomass by the disturbance. The mortality of temperature-sensitive microbes has been observed in other studies where heat and combined heat-drought stresses were applied (Bérard et al., 2011; Guillot et al., 2019; Hamdi et al., 2011). The effect of temperature on SIR activities was also evident when comparing the heat stress responses of planted soils. In our study, L soils presented the highest values of SIR activity under control conditions, but after heat stress, SIR activity decreased to levels comparable to the other treatments. Then, we concluded that an important microbial biomass in L soils did not confer a better resistance to the heat disturbance. This finding was consistent with Guillot et al. (2019), who observed that Mediterranean soils exhibiting the highest microbial biomass were not systematically

associated with a higher resistance. Conversely, W soils which exhibited the lowest values of SIR, presented the greatest resistance index (Table 2), which suggests a greater proportion of more heat-tolerant microbes within this modality. A weaker plant legacy effect on C-related microbial activities due to inherent quality of this type of soil (Salomé et al., 2014) was expected. However, recent studies pointed out that combined heat and drought stresses were able to significantly alter the microbial biomass (Bérard et al., 2011; Guillot et al., 2019), or acclimate of soil communities to heat waves (Bérard et al., 2012) in Mediterranean soils. In our study, the plant-induced effects may be partly due to a soil microbes protection by plant liberation of exopolysaccharides (Kumar and Verma, 2018). Soils with L and WL legacies presented the highest resilience indices and recovery rates for SIR. The rapid progression of L and WL soils over time reduced final differences between these soils and W at T28 (Fig. 2). Despite strongly affected by heat stress, the better resilience of L and WL suggest that heat tolerant microbes rapidly proliferated. The greater resilience of L soils could be attributed to a greater C availability derived from death of the most sensitive microorganisms (Bérard et al., 2015), but also by transformation of soil organic matter as induced by the elevated temperature (Bérard et al., 2011; Hamdi et al., 2011). It should be noted that a positive priming effect due to lupin legacy may also contribute to C availability in L soils conferring a greater resilience of L and WL (Wang et al., 2016).

4.3. Effects of experimental conditions on N-related microbial activities: Nitrification Enzyme Activity (NEA)

NEA was affected by heat stress, but to a lesser extent than SIR, while comparable to BR (Fig. 2). Both temperature and moisture are considered as major environmental factors affecting microbial activities and structure. Considerable debate stands in literature about optimal temperature for soil nitrifiers (Taylor et al., 2017) with scarce studies dealing with the

effect of nitrification rate at temperatures higher than 40°C. Indigenous nitrifiers have different optimal temperatures depending on native region (Avrahami and Conrad, 2003). Albeit > 40°C temperatures may be experienced in Mediterranean regions, the duration of continuous heat stress, *i.e.* 2 days, is not reproducible under Mediterranean field conditions. French regulation for Mediterranean region defines severe heat waves as a period lasting 2-3 days, and characterized by elevated temperature > 35°C and > 21°C during day and night respectively. Thus, night cooling decreases temperature stress even during severe heat waves, which was not the case during our experiment. Then, 2 days on continuous applied 48°C temperature have to be considered as a stress while not fully realistic of field conditions. One should note our moisture content of soils (100% of WHC) during the experiment. Nitrification rate is affected by drying-rewetting cycles (Fierer and Schimel, 2002) such as mineralization (Zhang et al., 2017). Fierer and Schimel (2002) depicted an increase of nitrification potentials in frequently drying-rewetting stress treatments. In their experiment, they do not succeed to attribute any changes of nitrification rates when soil moisture increased from 35% to 50% WHC. Mathieu et al. (2006) estimated no significant differences of nitrification rates using ¹⁵N tracer under 150% WHC (saturated) and 75% of WHC (unsaturated conditions) in soil incubations with 2 mm aggregates. We thus assumed that our uncommon WHC did not alter potential nitrification rates. However, possible restricted oxygen availability due to O₂ consumption by mineralization during the incubation period could be pointed out. A O₂ restriction may finally alter nitrifier's community growth rate and activity at earlier dates of our experiment. As nitrification potentials are estimated under shaking to provide aeration, oxygen limitation at earlier dates (T2 and T7) may have been less pronounced than at T16. One should note that our goal was to test if planted treatments conferred different legacy effects on potential NEA in response to a heat disturbance. Despite discussed limitations of our experiment, we discussed significant differences while keeping in mind potential

limitations of field transposition of our results. We demonstrated that WL legacy exhibited comparable resistance and resilience than those with W legacy. The WL soil was the less affected by the heat stress initially (T2), with a reduction by only 31% in NEA relative to the control (Fig. 2), but this trend was not reflected on resistance indices, probably due to the high variability among replicates. However, in terms of resilience, at T16, significant differences between indices were verified, with WL and W soils exhibiting the highest resilience indices. Pivato et al. (2017) estimated a greater abundance of nitrifiers in soils from plant associations, whereas Le Roux et al. (2013) and Zhang et al. (2015) reported a negative effect of plant richness on the abundance of ammonia oxidizers. These latter articles suggested that plant identity rather than plant diversity can affect nitrifier community abundances. Considering gene-based associated groups of nitrifiers namely Ammonia Oxidizing Archaea and Bacteria (AOA and AOB), Thion et al. (2016) estimated greater AOA abundance in the rhizosphere of exploitative grass species, like wheat. Moreover, Taylor et al. (2017) considered that AOA nitrifiers are more tolerant to higher temperatures. Thus, better resilience in planting modalities containing wheat could be attributed to these published results. Finally, one should also note the interesting NEA dynamic observed at later stages in our study. A strong increase in NEA activities of all soils was observed at T28, compared to T16, for both control and heat stress conditions (Table 1). Such increase in NEA at this final date could be explained by a “boost” in the activities of nitrifiers after 30 days of incubation in optimal conditions. Since the growth rates of nitrifiers are recognized to be relatively slow (Robertson and Groffman, 2015; Wertz et al., 2007), the interpretation of NEA resilience is then highly dependent of the final experimental dates. Accordingly, one should note that individual properties of microorganisms like dormancy, plasticity and growth rate (Shade et al., 2012) are also important components of resistance and resilience of soil microbial communities.

Conclusions

Adaptation to climate change of Mediterranean soils regularly exposed to heat waves may be possible through plant legacy effects. Intercropping confers more stability of soil microbial activities compared to single crop species when both C and N are investigated together due to individual benefits of each plant type. Finally, additional studies are essential to investigate if, i) prolonged plant presence will allow a more persistent effect on soils, and if ii) longer period of incubation would be able to detect full recovery of soil microbial activities over time. These results can provide useful information for designing cropping systems, in particular crop rotations and/or intercropping to capitalize on different legacy effects of various crops.

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Figure and table captions

Fig. 1. Principal component analysis (PCA) performed on basal respiration (BR), substrate-induced respiration (SIR), nitrification enzyme activity (NEA) and soil mineral N content as nitrate (N-NO_3^-) and ammonium (N-NH_4^+). Symbols stand for planting legacy (W – wheat, L – lupin, WL – wheat + lupin, U – unplanted) (a) and sampling dates (T2, T7, T16 and T28 – 2, 7, 16 and 28 days after the end of the heat stress) within control (C) and heat stress (S) conditions (b). Centroids are represented by larger symbols.

Fig. 2. Percentages of change in microbial activities (BR – basal respiration, SIR – substrate-induced respiration, NEA – nitrification enzyme activity) relative to controls along all sampling dates after the end of the heat stress (T2, T7, T16, T28). Symbols represent planting legacy (W – wheat, L – white lupin, WL – wheat + lupin, U – unplanted). Vertical bars indicate standard errors ($n=4$). Asterisks indicate significant differences between planting legacies within a sampling date (one-way ANOVA, followed by a Tukey post hoc test ($p < 0.05$)).

Fig. 3. Redundancy analysis (RDA) based on resistance (RS) (a) or resilience (RL) (b) indices of basal respiration (BR), substrate-induced respiration (SIR) and nitrification enzyme activity (NEA), constrained by BR, SIR, NEA, as well as soil mineral N content as nitrate (N-NO_3^-) and ammonium (N-NH_4^+). Resistance indices were calculated for T2, whereas resilience indices were calculated for T16 (2 and 16 days after the end of the heat stress). Groups of planting legacies are represented by W (wheat) in blue, L (lupin) in orange, WL (wheat + lupin) in green and U (unplanted) in red. The RDA was performed on center-reduced matrix.

Table 1. Basal respiration (BR – in $\mu\text{g C-CO}_2 \text{ day}^{-1} \text{ g}^{-1}$ dry soil), substrate-induced respiration (SIR – in $\mu\text{g C-CO}_2 \text{ h}^{-1} \text{ g}^{-1}$ dry soil) and nitrification enzyme activity (NEA – in $\mu\text{g N-NO}_2^- + \text{N-NO}_3^- \text{ h}^{-1} \text{ g}^{-1}$ dry soil) of each planting legacy (W – wheat, L – lupin, WL – wheat+lupin, U – unplanted), within control and stress conditions, for each sampling date after the end of the heat stress (T2, T7, T16, T28). Values are the means of four replicates ($n=4$). Thresholds of probability of the two-way ANOVA (Planting x Stress) are indicated at the top of each variable-related data set. When significant interaction between factors (Planting x Stress) is present, different bold letters represent significant differences between 8 means (i.e. control and heat stress conditions combined; Tukey post hoc test ($p < 0.05$)). Different lowercase letters represent significant differences within a single column, whereas different uppercase letters represent either significant differences between stress conditions (Control vs Stress), or between planting legacies with control and stress conditions combined (Tukey post hoc test ($p < 0.05$)).

Table 2. Indices of resistance and resilience based on basal respiration (BR), substrate-induced respiration (SIR) and nitrification enzyme activity (NEA) for each specific plant legacy (W – wheat, L – lupin, WL – wheat+lupin). Resistance indices were calculated on T2 date (2 days after the end of the heat stress), whereas resilience indices were calculated on T7, T16 and T28 dates of measure (7, 16 and 28 days after the end of the heat stress). Values are the means of four replicates ($n=4$). Probabilities of the one-way ANOVA are indicated at the bottom of each variable-related data set. Different letters represent significant differences within a column (Tukey post hoc test ($p < 0.05$)).

Fig. 1. Principal component analysis (PCA) performed on basal respiration (BR), substrate-induced respiration (SIR), nitrification enzyme activity (NEA) and soil mineral N content as nitrate (N-NO_3^-) and ammonium (N-NH_4^+). Symbols stand for planting legacy (W – wheat, L – lupin, WL – wheat + lupin, U – unplanted) (a) and sampling dates (T2, T7, T16 and T28 – 2, 7, 16 and 28 days after the end of the heat stress) within control (C) and heat stress (S) conditions (b). Centroids are represented by larger symbols.

Fig. 2. Percentages of change in microbial activities (BR – basal respiration, SIR – substrate-induced respiration, NEA – nitrification enzyme activity) relative to controls along all sampling dates after the end of the heat stress (T2, T7, T16, T28). Symbols represent planting legacy (W – wheat, L – white lupin, WL – wheat + lupin, U – unplanted). Vertical bars indicate standard errors ($n=4$). Asterisks indicate significant differences between planting legacies within a sampling date (one-way ANOVA, followed by a Tukey post hoc test ($p < 0.05$)).

Fig. 3. Redundancy analysis (RDA) based on resistance (RS) (a) or resilience (RL) (b) indices of basal respiration (BR), substrate-induced respiration (SIR) and nitrification enzyme activity (NEA), constrained by BR, SIR, NEA, as well as soil mineral N content as nitrate (N-NO_3^-) and ammonium (N-NH_4^+). Resistance indices were calculated for T2, whereas resilience indices were calculated for T16 (2 and 16 days after the end of the heat stress). Groups of planting legacies are represented by W (wheat) in blue, L (lupin) in orange, WL (wheat + lupin) in green and U (unplanted) in red. The RDA was performed on center-reduced matrix.

Variable	Factor	T2		T7		T16		T28	
BR	Planting legacy	< 0.001***		< 0.001***		< 0.05*		ns	
	Heat stress	< 0.01**		< 0.001***		< 0.05*		ns	
	Planting x Stress	ns		ns		ns		ns	
		Control ^B	Stress ^A	Control ^B	Stress ^A	Control ^B	Stress ^A	Control	Stress
	W	32.82 ab	41.39 ab ^{BC}	21.52 b	29.79 bc ^B	20.18 a	22.73 a ^{AB}	20.07 a	19.31 a
	L	41.61 a	63.79 a ^A	37.91 a	48.14 a ^A	21.79 a	24.76 a ^A	20.19 a	23.88 a
	WL	39.32 a	43.41 ab ^{AB}	21.56 b	38.46 ab ^B	20.99 a	24.37 a ^A	15.78 a	18.37 a
	U	23.73 b	33.23 b ^C	18.63 b	25.30 c ^B	16.17 a	19.21 a ^B	16.50 a	16.12 a
	Planting legacy	< 0.001***		< 0.001***		< 0.001***		< 0.001***	
	Heat stress	< 0.001***		< 0.001***		< 0.001***		< 0.001***	
SIR	Planting x Stress	< 0.001***		< 0.001***		< 0.001***		< 0.01**	
		Control	Stress	Control	Stress	Control	Stress	Control	Stress
	W	5.77 c	2.01 d	4.33 c	1.65 e	4.21 bc	2.64 de	4.94 bc	3.15 de
	L	12.42 a	2.59 d	9.11 a	2.10 e	7.69 a	2.75 de	6.92 a	4.00 cd
	WL	9.24 b	2.78 d	6.56 b	2.20 de	5.38 b	2.41 de	5.38 b	3.43 de
	U	4.98 c	1.72 d	3.50 cd	1.71 e	3.47 cd	1.81 e	3.82 d	2.67 e
	Planting legacy	< 0.001***		< 0.001***		< 0.001***		< 0.001***	
	Heat stress	< 0.001***		< 0.001***		ns		< 0.05*	
	Planting x Stress	ns		ns		ns		< 0.001***	
		Control ^A	Stress ^B	Control ^A	Stress ^B	Control	Stress	Control	Stress
NEA	W	0.54 b	0.16 b ^B	0.40 b	0.15 b ^B	0.59 b	0.30 b ^B	0.65 c	2.69 bc
	L	0.54 b	0.17 b ^B	0.57 ab	0.12 b ^B	0.74 b	1.12 ab ^B	2.83 bc	4.66 b
	WL	0.42 b	0.28 b ^B	0.40 b	0.11 b ^B	0.43 b	0.45 ab ^B	1.28 c	2.63 bc
	U	1.44 a	1.21 a ^A	0.91 a	0.49 a ^A	1.99 a	1.36 a ^A	8.63 a	5.38 b
	Planting legacy	< 0.001***		< 0.001***		< 0.001***		< 0.001***	

Table 1. Basal respiration (BR – in $\mu\text{g C-CO}_2 \text{ day}^{-1} \text{ g}^{-1}$ dry soil), substrate-induced respiration (SIR – in $\mu\text{g C-CO}_2 \text{ h}^{-1} \text{ g}^{-1}$ dry soil) and nitrification enzyme activity (NEA – in $\mu\text{g N-NO}_2^- + \text{N-NO}_3^- \text{ h}^{-1} \text{ g}^{-1}$ dry soil) of each planting legacy (W – wheat, L – lupin, WL – wheat+lupin, U – unplanted), within control and stress conditions, for each sampling date after the end of the heat stress (T2, T7, T16, T28). Values are the means of four replicates (n=4). Thresholds of probability of the two-way ANOVA (Planting x Stress) are indicated at the top of each variable-related data set. When significant interaction between factors (Planting x Stress) is present, different bold letters represent significant differences between 8 means (i.e. control and heat stress conditions combined; Tukey post hoc test ($p < 0.05$)). Different lowercase letters represent significant differences within a single column, whereas different uppercase letters represent either significant differences between stress conditions (Control vs Stress), or between planting legacies with control and stress conditions combined (Tukey post hoc test ($p < 0.05$)).

Variable	Plant legacy	T2 (Resistance)	T7	T16 (Resilience)	T28
BR	W	0.52	- 0.00	0.27	0.48
	L	0.34	0.31	0.55	0.27
	WL	0.60	- 0.22	0.20	0.26
	<i>p-value (one-way ANOVA)</i>	<i>0.61</i>	<i>0.31</i>	<i>0.61</i>	<i>0.86</i>
SIR	W	0.21 a	0.16	0.47	0.35 b
	L	0.11 b	0.16	0.32	0.54 a
	WL	0.17 ab	0.19	0.37	0.53 a
	<i>p-value (one-way ANOVA)</i>	<i>0.05*</i>	<i>0.87</i>	<i>0.63</i>	<i>0.04*</i>
NEA	W	0.17	0.21	0.24 a	- 0.63
	L	0.20	-0.03	- 0.58 b	- 0.48
	WL	0.29	- 0.10	0.46 a	- 0.68
	<i>p-value (one-way ANOVA)</i>	<i>0.34</i>	<i>0.16</i>	<i>0.008**</i>	<i>0.56</i>

Table 2. Indices of resistance and resilience based on basal respiration (BR), substrate-induced respiration (SIR) and nitrification enzyme activity (NEA) for each specific plant legacy (W – wheat, L – lupin, WL – wheat+lupin). Resistance indices were calculated on T2 date (2 days after the end of the heat stress), whereas resilience indices were calculated on T7, T16 and T28 dates of measure (7, 16 and 28 days after the end of the heat stress). Values are the means of four replicates (n=4). Probabilities of the one-way ANOVA are indicated at the bottom of each variable-related data set. Different letters represent significant differences within a column (Tukey post hoc test ($p < 0.05$)).