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.

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

75   al., 2015; Doré et al., 2011; Malézieux et al., 2009). Long-term legacy effects of intercropping

76   have been estimated by Cong et al. (2015), but within 1-year timespan authors are reluctant to

77   expect a significant effect on soil communities. However, Pivato et al. (2017) estimated that

78   after only 12 weeks of bi-species culture, N cycling-related microbial communities were

79   strongly affected especially at low N level compared to each species in monoculture. Grman

80   and Suding (2010) also estimated a short-term legacy effect pointing out the multiple drivers

81   defining both plant legacy duration and intensity. Given the crucial role of soil microbial

82   activities in ecosystem functioning, these plant legacy effects can contribute to agroecosystem

83   stability in the context of climate change and particularly during extreme events. Several

84   studies have been focused on resistance (capacity to withstand change) and resilience

85   (capacity for recovering of function) of C-related soil microbial activities to environmental

86   disturbances (Bérard et al., 2012; Bérard et al., 2011; Chaer et al., 2009; Guillot et al., 2019;

87   Hamdi et al., 2011; Mooshammer et al., 2017; Orwin and Wardle, 2005). However, studies

88   about stability of more specialized soil microbial communities like nitrifiers are scarce

89   (Mooshammer et al., 2017; Thion and Prosser, 2014; Wertz et al., 2007). Yet there are

90   reasons to argue that plants are able to shape soil related N-cycle communities (Zak et al.,

91   2003). Our rationale is to consider that diverse plant species yield different legacies,

92   ultimately contributing to alleviate effects of extreme climatic events as heat waves in

93   agroecosystems. We hypothesized that intercropped species promote a (i) different legacy

94   effect on soil microbial processes in comparison to single species, and accordingly (ii)

95   influence the resistance and resilience of soils. We focused on nitrification and respiration

96   microbial processes (potential nitrification, substrate-induced respiration and basal

97   respiration) as key processes of the N and C cycles in environmental and plant productivity

98   issues. To achieve this goal, we generated a novel plant legacy in a calcareous cambisol,

99   through a greenhouse experiment conducted with a soil collected in a Mediterranean site near

100   Montpellier, France. Soil was collected 3 months after a pea crop, sieved at 1-cm to be

101   homogenized and ridded of any coarse organic material, then air dried and stored at ambient

102   temperature before use. In 2017, soil was mixed with perlite and conditioned by one complete

103   vegetative cycle of two crop species grown as single crops (monocultures), (i) white lupin

104   (*Lupinus albus* L.) and (ii) bread wheat (*Triticum aestivum* L.), as well as the mixture of both

105   species (intercrop). Finally, we calculated the resistance and resilience indices (*sensu* Orwin

106   and Wardle (2004)) after a heat disturbance.

107

108   **2. Material and methods**

109   **2.1. Soil origin and greenhouse experiment before heat stress experiment**

110   Soil originated from a plot of the INRA experimental station localized at Mauguio

111   (3°59'6"E, 43°37'13"N, 12 m altitude) in French littoral Mediterranean region. The soil was a

112   calcareous cambisol with a clay loam texture according to USDA classification (clay 294 g

113   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}

114   ), which had developed on an alluvial calcareous parent material, typical of the South East

115   plains of Montpellier. The regional climate is typically Mediterranean with surface regularly

116   exposed to drying-rewetting cycles due to irregular rain events mainly concentrated in

117   Autumn and Spring. The annual average rainfall at this site during the growing season, from

118   September to June, over the last 20 years was 598 mm. Recorded average soil temperatures at

119   10 cm depth since 2012 demonstrated that plot had never experienced temperatures higher

120   than 38.4 °C. During the four months before soil sampling, 3 cycles of drought were recorded

121   (*i.e.* 20 days with cumulative rainfall lower than 2mm, according to Tang et al. (2014)), while

122   maximal air temperatures ranged from 22.8 °C (in September) to 34.7 °C (in August). The last

123   precipitation episode (4.8 mm) preceding soil sampling occurred 5 days earlier. Soil was

124   collected (0-15 cm depth) 3 months after pea harvest, air dried and sieved at 1-cm to

125   homogenize and remove coarse organic material, then finally stored at ambient temperature

126   before use to preserve inherent soil quality (Salomé et al., 2014).

127   A pot experiment was then conducted in a greenhouse (Centre Mondial de

128   l'Innovation – Roullier Group – Saint-Malo, France) from September to December 2017 with

129   the soil previously described. Before being conditioned in the pots (4-dm^3), the soil was re-

130   sieved with a 4-mm mesh to remove any organic material and mixed with perlite to allow

131   better conditions for plant root development. Pots were filled with substrate composed of 2 kg

132   of soil and 0.2 kg of perlite. The characteristics of the substrate were clay 202 g kg^{-1}, silt 454

133   g kg^{-1}, sand 343 g kg^{-1}, total CaCO$_3$ 39 g kg^{-1}, pH$_w$ 8.3, CEC$_{Metson}$ 15 cmol$_+$ kg^{-1}, organic C

134   10.4 g kg^{-1}, total N 0.95 g kg^{-1}, ammonium (N-NH$_4^+$) 20 mg kg^{-1}, nitrate (N-NO$_3^-$) 39 mg kg^{-1}.

135   The WHC of the substrate corresponded to 28% of gravimetric humidity. For simplicity,

136   substrate will be named herein as soil. The planting modalities of this experiment were: (i)

137   monoculture of wheat (W) (*Triticum aestivum* L. cv. Lennox) at a density of 4 plants per pot,

138   (ii) monoculture of white lupin (L) (*Lupinus albus* L. cv. Feodora) at a density of 2 plants per

139   pot, (iii) intercropping of both species (WL) at half of the density for each corresponding

140   monoculture (2 wheat and one lupin plants), and (iv) an unplanted soil (U). Each planted and

141   unplanted treatments was replicated 4 times. At maturity, plants (shoots and roots) were

142   harvested and the soil was carefully separated from the roots by gently shaking followed by

143   hand sorting and then kept at 4°C in the dark until microcosm experiment (16 weeks).

144

2.2. Heat stress microcosm experiment

146   A total of 128 microcosms were prepared (4 replicates x 4 planting treatments x 2

147   temperatures x 4 sampling dates) containing 50 g of equivalent dry soil from greenhouse

148   experiment, and placed in 1-dm^3 glass jars hermetically sealed by a rubber gasket. Soils of

149   microcosms were rehydrated at 100% of the water holding capacity and pre incubated at 12°C

150   for 5 days in order to ensure uniform initial experimental conditions. Afterwards, half of the

151   microcosms (64 samples) were incubated at 28°C and the remaining half at 48°C,

152   corresponding to control (C) and heat stress (S) conditions, respectively. All microcosms were

153   placed into darkness in laboratory incubators (KBWF 720, Binder, Germany) with a precision

154   of 0.4 °C. In order to i) avoid any drying-rewetting cycles during the experiment altering the

155   C and N cycles including nitrification (Fierer and Schimel, 2002) and ii) ensure precise

156   estimation of nitrification potential assays conducted with soil slurries (see section 2.3), we

157   kept soil moisture at 100% WHC. To ensure no variations of water regime in microcosms, a

158   20-cm^3 polypropylene container filled with water was placed in each sealed microcosm.

159   Microcosms were regularly weighted to check any soil moisture changes. After two days of

160   heat disturbance at 48°C, the stressed microcosms were transferred back at 28°C. Each

161   microcosm contained an 1M NaOH trap for basal respiration determination (see section 2.3).

162   At each sampling date (T2, T7, T16 and T28 corresponding to 2, 7, 16 and 28 days after the

163   end of the heat stress), samples were immediately split into 4 subsamples for all subsequent

164   soil analyses (substrate-induced respiration (SIR), nitrification enzyme activity (NEA), soil

165   NH_4^+ and NO_3^- measurements, and soil water content). Measurements of SIR and NEA were

166   performed on fresh soils within 2 days after sampling, whereas soil samples for mineral N

167   measurements were frozen (-20°C) until analysis.

168

169   **2.3. Soil microbial and chemical analyses**

170   Basal respiration (BR) traps were collected and replaced at each sampling date. An

171   aliquot of 1-cm^3 of NaOH was back-titrated with 0.1 M HCl, after adding 2.5-cm^3 of $BaCl_2$

172   solution (30 %) to precipitate the Na_2CO_3 issued from soil CO_2 respiration. Results of BR

173   were expressed in µg C-CO$_2$ day^{-1} g^{-1} of dry soil. SIR measurements were performed on 10 g

174   of equivalent dry soil according to Patra et al. (2005), in presence of glucose (1.2 mg C-

175   glucose g^{-1} dry soil). A CO_2 accumulation kinetic was measured using a gas chromatograph

176   (µGC R3000, SRA instruments, France) at 28°C. SIR was expressed in µg $C-CO_2$ h^{-1} g^{-1} of

177   dry soil. NEA was measured with 3 g of equivalent dry soil according to Patra et al. (2005).

178   Briefly, a $(NH_4)_2SO_4$ solution (50 µg $N-NH_4^+$ g^{-1} dry soil) was added to each soil sample.

179   Samples were placed at 28°C, shaken at 140 rpm to ensure aerobic conditions during 10

180   hours, and NO_2^- + NO_3^- production was measured every two hours using a photometer

181   (Smartchem 200, AMS Alliance, France). NEA was expressed in µg of $N-(NO_2^-+NO_3^-)$ h^{-1} g^{-1}

182   of dry soil. Finally, soil ammonium ($N-NH_4^+$) and soil nitrate ($N-NO_3^-$) were extracted from a

183   soil:solution ratio of 1:2 with 1M KCl solution after a 30 min of contact at 140 rpm, followed

184   by 30 min of rest. Supernatant were filtered at 0.45 µm and mineral N concentrations were

185   estimated with an Analytical Discrete Multi-Chemistry Analyzer (AQ2, SEAL Analytical,

186   UK). Soil mineral N content were expressed as mg $N-NH_4^+$ and mg $N-NO_3^-$ kg^{-1} of dry soil.

187

188   **2.4. Percentage change relative to control**

189   In order to investigate recovery rate, percentage change of values in stressed samples

190   relative to those in the controls, at each sampling date, were calculated as follows (Bérard et

191   al., 2011; Chaer et al., 2009):

192

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

194

195   where S_i and C_i are the variable values in the stressed and control soil samples, respectively,

196   at sampling date i (*i.e.* T2, T7, T16 or T28).

197

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):

$$RS = 1 - \frac{2 \times |D_2|}{C_2 + |D_2|} \tag{2}$$

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;

$$RL = \frac{2 \times |D_2|}{|D_2| + |Dx|} - 1 \tag{3}$$

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

222 response variable has not fully recovered, whereas a negative index value indicates that the

223 absolute value of D_x is higher than the absolute value of D_2 (*i.e.* the soil has low resilience).

224

225 **2.6. Statistical analyses**

226 The effects of planting legacy (*i.e.* both planted (W, L, WL) and unplanted (U)

227 modalities) and heat stress on all soil variables were tested for each sampling date by two-way

228 ANOVA (planting legacy x heat stress). Afterwards, one-way ANOVA was performed in

229 absence of interaction to test differences among treatments. The effect of specific plant legacy

230 (*i.e.* only planted modalities – W, L, WL) on resistance and resilience indices, as well as the

231 effect of planting legacy on percentages of change in microbial activities relative to controls

232 were also tested by one-way ANOVA, within each sampling date. Significant differences

233 between means were tested by Tukey's multiple comparison tests ($p < 0.05$). Normality of

234 residuals and homogeneity of variance were tested by the Shapiro and Levene's tests,

235 respectively. When necessary, data for microbial activities were squared root-transformed.

236 Principal component analysis (PCA) were performed with all chemical and microbial soil

237 variables. Within a PCA, coordinates of the individuals on each axis were used for testing

238 differences between groups (*i.e.* levels of a factor) by one-way ANOVA or Kruskal-Wallis

239 tests in case of non-normality. Tukey's multiple comparison tests ($p < 0.05$) or Wilcoxon rank

240 sum tests ($p < 0.05$) were then performed accordingly. Finally, a redundancy analysis (RDA)

241 was performed to assess the effect of all soil microbial and chemical variables (SIR, NEA,

242 BR, $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ contents) on resistance (at T2) and resilience (at T16) indices.

243 Statistical analyses were performed with R software v. 3.5.1 (R Core Team, 2018). Packages

244 used for the ANOVA, Tukey's test, PCA and RDA analysis were: *car*, *agricolae*,

245 *FactoMineR* and *vegan*, respectively.

246

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, $N-NH_4^+$, $N-NO_3^-$ measurements for all sampling dates and stress conditions) explained 74.2% of the total variance (Fig. 1). BR, $N-NH_4^+$, NEA and $N-NO_3^-$ 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

270   Planting legacy was significant for BR values at the first two dates of measure ($p <$

271   0.001; Table 1 and Fig C.1). At these dates, L soils clearly differed from unplanted soils, with

272   L soils showing values 1.9-fold greater, when combining control and stress conditions. The

273   planting legacy was still significant at T16, but no effect was observed at T28. An important

274   effect of heat stress was also observed on BR values, particularly at T2 and T7 ($p < 0.01$ and p

275   < 0.001, respectively). At the two first dates, stressed soils presented values 1.35-fold greater

276   than control soils on average. At T16 the difference between stressed and control soils was

277   lower, whereas no significant difference between stressed and control soils was observed at

278   T28.

279   In regard to SIR, planting legacy ($p < 0.001$), heat stress ($p < 0.001$) and planting x

280   stress interaction ($p < 0.01$ at T28 and $p < 0.001$ at other dates) had a strong effect on SIR

281   values (Table 1 and Fig. C.2). Significant differences between planting legacies of control

282   soils were observed at all sampling dates. However, heat stress alleviated these differences.

283   For instance, at T2 (control), SIR means ranked as follows: L > WL > W = U, whereas no

284   differences were noticed after stress. One should note that L soils exhibited the greatest SIR

285   values, while unplanted soils presented the lowest. Conversely to BR, stressed conditions

286   exhibited a dramatic decrease of SIR activities compared to the controls, whatever the

287   sampling date. The most important difference was observed at the first date, when stressed

288   soils were 3.6-fold lower than controls. The L soils were particularly altered at the first date

289   because SIR activity were 5-fold lower than controls. This difference between stress and

290   control conditions was also present at the other dates, but decreased with time.

291   A very strong effect of planting legacy on NEA values was observed, whatever the

292   sampling date ($p < 0.001$; Table 1 and Fig. C.3). Unplanted soils exhibited the greatest values

293   compared to planted soils (2.2- to 3.8-fold greater than W, L and WL). The heat stress effect

294   on NEA was particularly observed at T2 and T7 ($p < 0.001$). At these dates, a significant

295   decrease in NEA values was observed between controls and stressed soils, but to a lesser

296   extent than that observed for SIR. At T16, heat stress had no effect on NEA, whereas at T28,

297   a significant effect of heat stress ($p < 0.05$) and interaction of heat stress and planting legacy

298   ($p < 0.001$) were observed. An interesting dynamic was observed from T16 to T28, as NEA

299   values increased particularly for stressed (S) soils, and to a lesser extent for control (C) soils.

300   Indeed, at T28, there was a final ranking with L values becoming closer to U values (U_C >

301   U_S = L_S ≥ L_C = W_S = WL_S > WL_C = W_C).

302

303   **3.3. Resistance and resilience of soil microbial activities**

304   Recovery rates are illustrated in Fig. 2. Just after heat disturbance (T2), SIR activity

305   was severely altered (-70%, on average) compared to NEA (-46%) and BR (+36%). The most

306   important decrease at T2 was observed for L soils, with a reduction of 79% in SIR relative to

307   control samples. At this date, L soil also exhibited the largest increase in BR (+59%) relative

308   to control samples. Conversely, for NEA, T2 showed the lowest decrease for the unplanted

309   soil (-15%). Focusing on the other dates for SIR, significant differences between percentages

310   of change were only observed at T7, as follows: U > W = WL > L (Table B.1). For NEA in

311   T16, percentages of change for WL and L, but not for W soils, switched from negative to

312   positive. Significant differences between values were observed for NEA at T28. The W soils

313   exhibited greater NEA values in stressed soils relative to controls and compared to WL and L

314   (Table B.1).

315   Indices of resistance (RS) and resilience (RL) are presented in Table 2. Significant

316   differences in resistance between soils were only observed for SIR ($p < 0.05$). The W soils

317   exhibited the highest RS index, which was significantly different from L, but not from WL.

318   For resilience indices, significant differences between soils for NEA at T16 ($p < 0.01$) and for

319   SIR at T28 ($p < 0.05$) were observed. The RL indices for NEA of both WL and W soils were

14

320   greater than that of L soils. The RL indices of L soils were negative at T7, T16, and T28,

321   meaning a very low resilience of NEA. For SIR at T28, the highest RL indices were found for

322   L and WL soils.

323   The ordination plots of RDA performed on resistance (RS), at T2, and resilience (RL),

324   only at T16, indices clearly discriminated the unplanted soil (Fig. 3 a,b). The resistance

325   indices of NEA were positively associated to NEA and $N-NO_3^-$ concentrations in soils, and

326   negatively to $N-NH_4^+$ contents. The RS indices of SIR were negatively associated to BR.

327   Considering resilience indices of NEA, these latter were negatively linked to NEA and N-

328   NO_3^-, whereas BR resilience indices were negatively linked to SIR and RL of SIR. Finally,

329   considering soils with only specific plant legacies (W, L, WL), L soils slightly distinguished

330   from the other soils on RDA plots for resistance, and strongly differed from the other soils for

331   resilience. Thus, these results confirmed percentages of change (Fig. 2) and RS and RL

332   indices (Table 2). The L soils were clearly different, being characterized by lower RS of SIR

333   and RL of NEA (Fig. 3 a,b).

334

335   **4. Discussion**

336   **4.1. Legacy effect of plants on soil microbial activities under control conditions**

337   Marked differences were observed between unplanted and planted soils, suggesting

338   that plants were responsible for a legacy effect. Unplanted soils always exhibited greater

339   values of NEA than those for W, L and WL. As plants are able to shape N-related microbial

340   communities (Moreau et al., 2015; Pivato et al., 2017), it occurred that all planted treatments

341   had a negative legacy effect on the activity of nitrifiers. This negative legacy effect may be

342   attributed to the competition between plants and microorganisms for N (Cantarel et al., 2015;

343   Kuzyakov and Xu, 2013; Xu et al., 2011). According to Schimel and Bennett (2004) plants

344   are effective competitors for ammonium which is also the substrate for nitrifiers, reducing

345　their abundance and activity. Some plant morphological and/or physiological characteristics

346　can also affect the diversity of nitrifiers (Cantarel et al., 2015; Legay et al., 2014; Pommier et

347　al., 2018), or inhibit nitrification (Subbarao et al., 2012). According to these recent results,

348　one should hypothesize that nitrifiers community is shaped by the environment (Erguder et

349　al., 2009; Prosser and Nicol, 2012; Zhalnina et al., 2012), or by plant N-use strategy (Thion et

350　al., 2016). Thereby, we can reasonably suggest a plant legacy effect on nitrification. A great

351　contrast between unplanted and planted soils was also observed for BR and SIR values, with

352　lower values of U compared to W, L and WL. This is consistent with Orwin and Wardle

353　(2005), who observed lower values of soil BR and SIR for bare soil compared to the planted

354　grass treatments. As large amounts of C-compounds can be released by roots as rhizodeposits,

355　e.g. sugars, organic acids, amino acids, mucilage, root or border cells (Kuzyakov and

356　Blagodatskaya, 2015; Philippot et al., 2013), an increase in microbial biomass and activities

357　(Tang et al., 2014) is expected in planted compared to unplanted soils.

358　　　　Considering only planted soils, we also observed different legacy effects among the

359　modalities (W, L, WL), which is consistent with our first hypothesis. Indeed, except for the

360　first sampling date, greater values of NEA were observed for L soil, compared to W and WL,

361　*i.e.* a stronger legacy effect for lupin than wheat. Le Roux et al. (2013) also reported a

362　positive relationship between the presence of legumes and NEA, which was explained by the

363　build-up of nitrifying communities in response to greater $N-NH_4^+$ availability in soils,

364　probably due to the release of N derived from Rhizodeposition (NdfR) by legumes (Duchene

365　et al., 2017). In addition, legumes are usually considered less competitive for soil mineral N

366　compared to cereals, which could suggest a higher availability of mineral N in soils under

367　legumes (Mallarino and Wedin, 1990). L soils were also remarkable regarding to C-related

368　microbial variables, showing the greatest values of SIR whatever the date of measurement, as

369　well as the highest values of BR at T2 and T7. White lupin is well known to exude large

370   quantities of organic anions, like citrate (Dissanayaka et al., 2015; Veneklaas et al., 2003;

371   Wang et al., 2010), compared to cereals and other legumes (Wang et al., 2016). Such exudates

372   may have promoted SIR and BR in our study. This positive legacy effect of white lupin on C-

373   related microbial variables compared to intercropped wheat and white lupin is however not in

374   agreement with the commonly observed positive effect of plant diversity on soil microbial

375   biomass and respiration (Chen et al., 2019; Eisenhauer et al., 2017; Tang et al., 2014).

376   Accordingly, one should note that legume legacies may be further investigated in

377   agroecosystems in order to decipher their potential role in rotation or in intercropping.

378

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

381   Amongst response variables, SIR was the most affected by heat stress (T2). Samples

382   affected by stress presented reductions varying from 65% to 79% relative to controls. As SIR

383   may be considered as a proxy of the active microbial biomass (Anderson and Domsch, 1978;

384   Orwin and Wardle, 2005), these results suggest a dramatic decrease in microbial biomass by

385   the disturbance. The mortality of temperature-sensitive microbes has been observed in other

386   studies where heat and combined heat-drought stresses were applied (Bérard et al., 2011;

387   Guillot et al., 2019; Hamdi et al., 2011). The effect of temperature on SIR activities was also

388   evident when comparing the heat stress responses of planted soils. In our study, L soils

389   presented the highest values of SIR activity under control conditions, but after heat stress, SIR

390   activity decreased to levels comparable to the other treatments. Then, we concluded that an

391   important microbial biomass in L soils did not confer a better resistance to the heat

392   disturbance. This finding was consistent with Guillot et al. (2019), who observed that

393   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 ^{15}N 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 O_2 consumption by mineralization during the incubation period could be pointed out. A O_2 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

444 limitations of field transposition of our results. We demonstrated that WL legacy exhibited

445 comparable resistance and resilience than those with W legacy. The WL soil was the less

446 affected by the heat stress initially (T2), with a reduction by only 31% in NEA relative to the

447 control (Fig. 2), but this trend was not reflected on resistance indices, probably due to the high

448 variability among replicates. However, in terms of resilience, at T16, significant differences

449 between indices were verified, with WL and W soils exhibiting the highest resilience indices.

450 Pivato et al. (2017) estimated a greater abundance of nitrifiers in soils from plant associations,

451 whereas Le Roux et al. (2013) and Zhang et al. (2015) reported a negative effect of plant

452 richness on the abundance of ammonia oxidizers. These latter articles suggested that plant

453 identity rather than plant diversity can affect nitrifier community abundances. Considering

454 gene-based associated groups of nitrifiers namely Ammonia Oxidizing Archaea and Bacteria

455 (AOA and AOB), Thion et al. (2016) estimated greater AOA abundance in the rhizosphere of

456 exploitative grass species, like wheat. Moreover, Taylor et al. (2017) considered that AOA

457 nitrifiers are more tolerant to higher temperatures. Thus, better resilience in planting

458 modalities containing wheat could be attributed to these published results. Finally, one should

459 also note the interesting NEA dynamic observed at later stages in our study. A strong increase

460 in NEA activities of all soils was observed at T28, compared to T16, for both control and heat

461 stress conditions (Table 1). Such increase in NEA at this final date could be explained by a

462 "boost" in the activities of nitrifiers after 30 days of incubation in optimal conditions. Since

463 the growth rates of nitrifiers are recognized to be relatively slow (Robertson and Groffman,

464 2015; Wertz et al., 2007), the interpretation of NEA resilience is then highly dependent of the

465 final experimental dates. Accordingly, one should note that individual properties of

466 microorganisms like dormancy, plasticity and growth rate (Shade et al., 2012) are also

467 important components of resistance and resilience of soil microbial communities.

468

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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704

Figure and table captions

706

707 **Fig. 1.** Principal component analysis (PCA) performed on basal respiration (BR), substrate-
708 induced respiration (SIR), nitrification enzyme activity (NEA) and soil mineral N content as
709 nitrate (N-NO$_3^-$) and ammonium (N-NH$_4^+$). Symbols stand for planting legacy (W – wheat, L
710 – lupin, WL – wheat + lupin, U – unplanted) (a) and sampling dates (T2, T7, T16 and T28 –
711 2, 7, 16 and 28 days after the end of the heat stress) within control (C) and heat stress (S)
712 conditions (b). Centroids are represented by larger symbols.

713 **Fig. 2.** Percentages of change in microbial activities (BR – basal respiration, SIR – substrate-
714 induced respiration, NEA – nitrification enzyme activity) relative to controls along all
715 sampling dates after the end of the heat stress (T2, T7, T16, T28). Symbols represent planting
716 legacy (W – wheat, L – white lupin, WL – wheat + lupin, U – unplanted). Vertical bars
717 indicate standard errors (n=4). Asterisks indicate significant differences between planting
718 legacies within a sampling date (one-way ANOVA, followed by a Tukey post hoc test ($p <$
719 0.05)).

720 **Fig. 3.** Redundancy analysis (RDA) based on resistance (RS) (a) or resilience (RL) (b) indices
721 of basal respiration (BR), substrate-induced respiration (SIR) and nitrification enzyme activity
722 (NEA), constrained by BR, SIR, NEA, as well as soil mineral N content as nitrate (N-NO$_3^-$)
723 and ammonium (N-NH$_4^+$). Resistance indices were calculated for T2, whereas resilience
724 indices were calculated for T16 (2 and 16 days after the end of the heat stress). Groups of
725 planting legacies are represented by W (wheat) in blue, L (lupin) in orange, WL (wheat +
726 lupin) in green and U (unplanted) in red. The RDA was performed on center-reduced matrix.

727 **Table 1.** Basal respiration (BR – in µg C-CO$_2$ day^{-1} g^{-1} dry soil), substrate-induced respiration
728 (SIR – in µg C-CO$_2$ h^{-1} g^{-1} dry soil) and nitrification enzyme activity (NEA – in µg N-NO$_2^-$ +
729 N-NO$_3^-$ h^{-1} g^{-1} dry soil) of each planting legacy (W – wheat, L – lupin, WL – wheat+lupin, U
730 – unplanted), within control and stress conditions, for each sampling date after the end of the
731 heat stress (T2, T7, T16, T28). Values are the means of four replicates (n=4). Thresholds of
732 probability of the two-way ANOVA (Planting x Stress) are indicated at the top of each
733 variable-related data set. When significant interaction between factors (Planting x Stress) is
734 present, different bold letters represent significant differences between 8 means (i.e. control
735 and heat stress conditions combined; Tukey post hoc test ($p < 0.05$)). Different lowercase
736 letters represent significant differences within a single column, whereas different uppercase
737 letters represent either significant differences between stress conditions (Control *vs* Stress), or
738 between planting legacies with control and stress conditions combined (Tukey post hoc test (p
739 < 0.05)).

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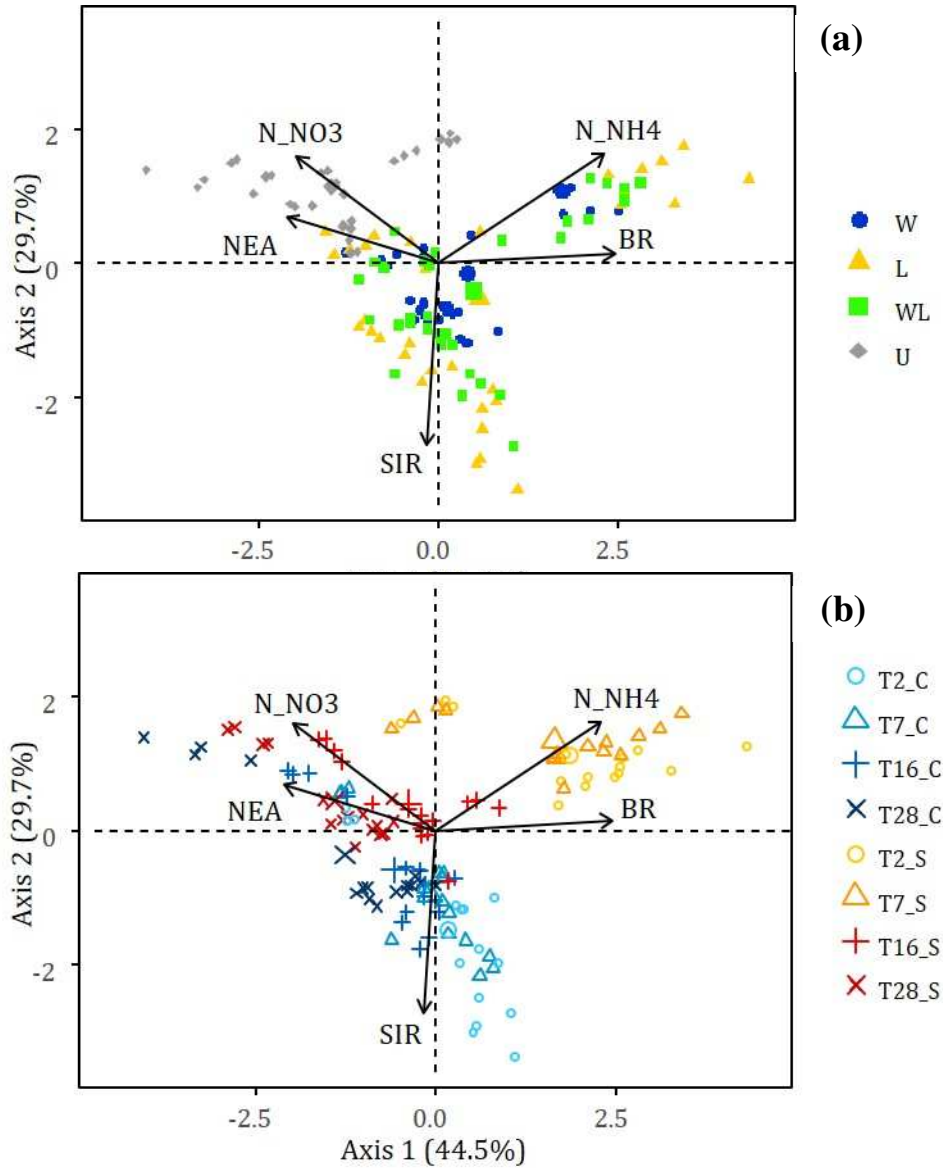747 within a column (Tukey post hoc test ($p < 0.05$)).

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.
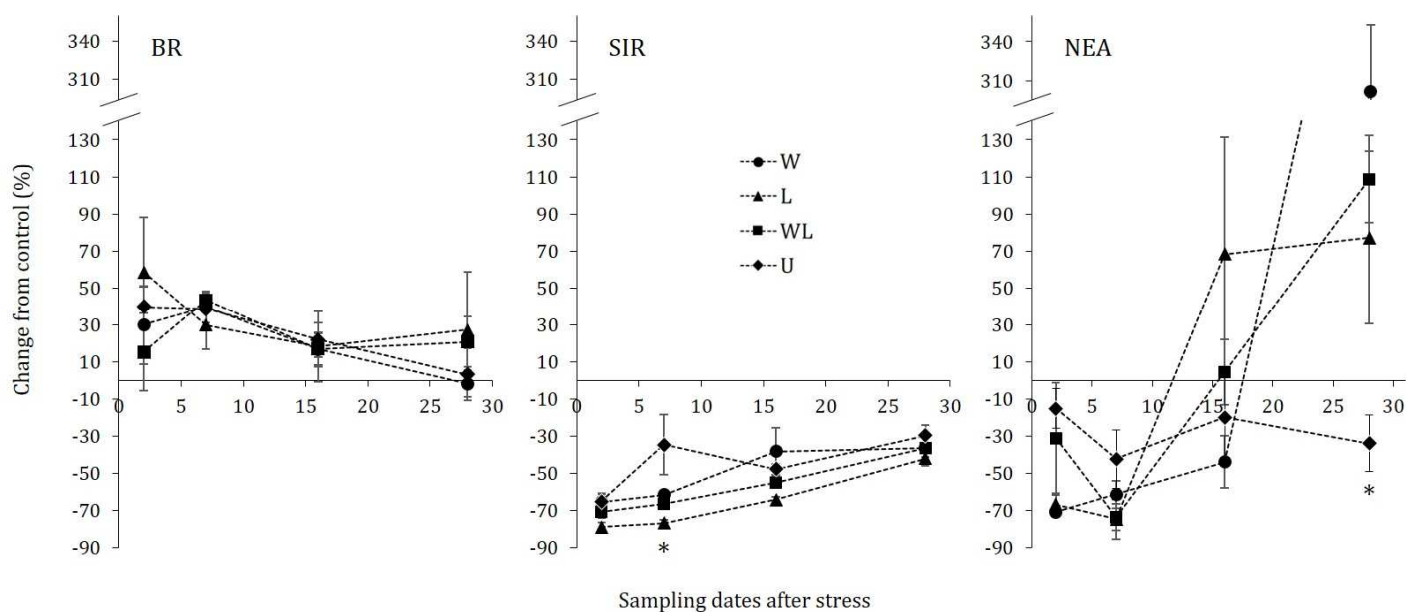
Fig. 2. Percentages of change in microbial activities (BR – basal respiration, SIR – substrate-induced respiration, NEA – nitrification enzyme activity) relative to controls along all sampling dates after the end of the heat stress (T2, T7, T16, T28). Symbols represent planting legacy (W – wheat, L – 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 ($p < 0.05$)).
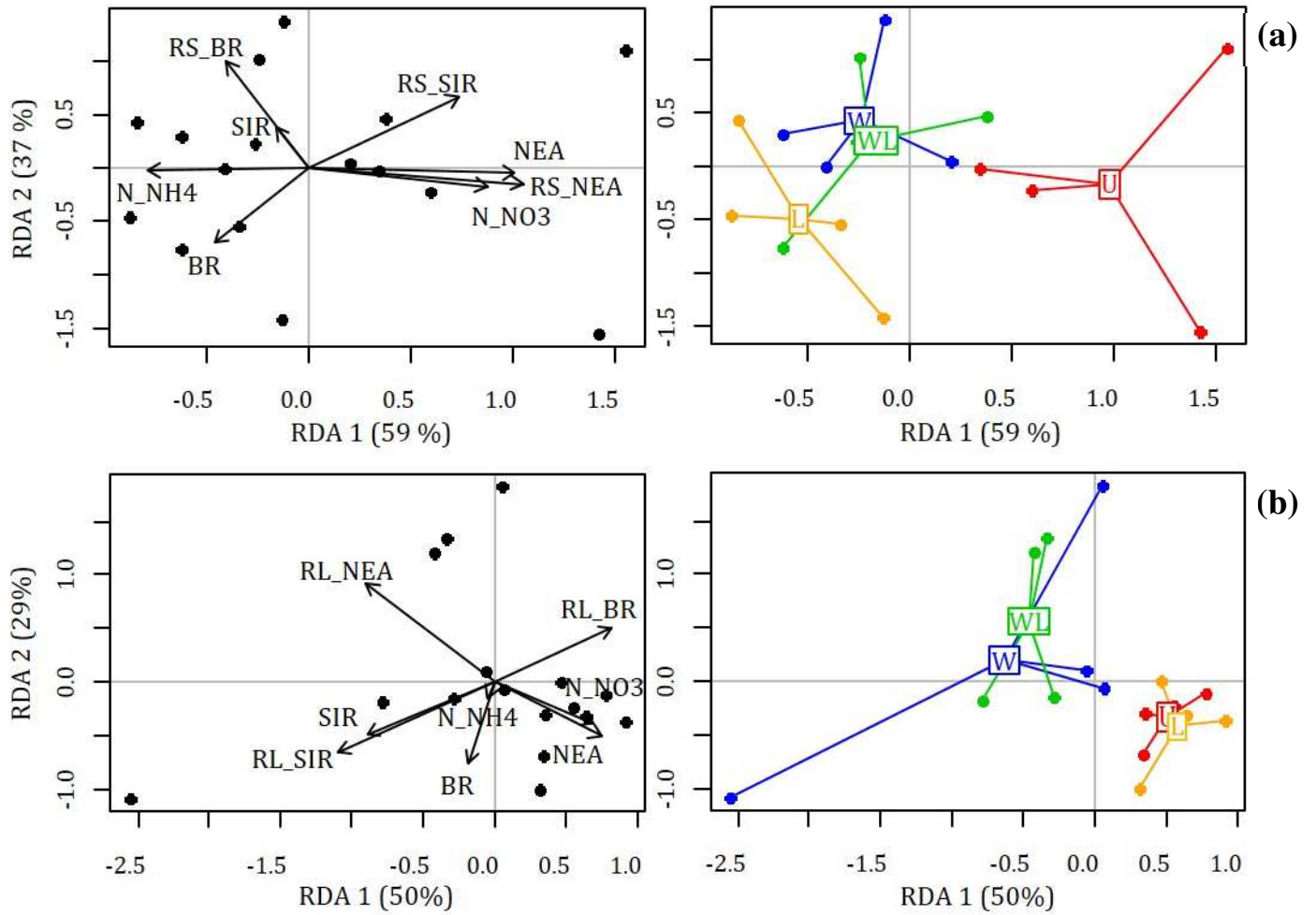
Fig. 3. Redundancy analysis (RDA) based on resistance (RS) (a) or resilience (RL) (b) indices of basal respiration (BR), substrate-induced respiration (SIR) and nitrification enzyme activity (NEA), constrained by BR, SIR, NEA, as well as soil mineral N content as nitrate (N-NO$_3^-$) and ammonium (N-NH$_4^+$). 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.

Table 1. Basal respiration (BR – in µg $C\text{-}CO_2$ day^{-1} g^{-1} dry soil), substrate-induced respiration (SIR – in µg $C\text{-}CO_2$ h^{-1} g^{-1} dry soil) and nitrification enzyme activity (NEA – in µg $N\text{-}NO_2^-$ + $N\text{-}NO_3^-$ h^{-1} 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).

Variable	Factor	T2			T7			T16			T28	
		Planting legacy										
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
SIR	Planting legacy	< 0.001***			< 0.001***			< 0.001***			< 0.001***	
	Heat stress	< 0.001***			< 0.001***			< 0.001***			< 0.001***	
	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**
NEA	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
	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**

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 (Resilience)	T16	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
p-value (one-way ANOVA)		*0.61*	*0.31*	*0.61*	*0.86*
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**
p-value (one-way ANOVA)		*0.05**	*0.87*	*0.63*	*0.04**
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
p-value (one-way ANOVA)		*0.34*	*0.16*	*0.008***	*0.56*

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 ($p < 0.05$)).