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Legume-barley intercropping stimulates soil N supply and crop yield in the succeeding durum wheat in a rotation under rainfed conditions

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Abstract

Legume-cereal intercropping is increasingly being appreciated in dryland areas, where severe climatic conditions and intensive agricultural practices, generally dominated by continuous cereal cultivation, determine depletion of soil nutrient resources and decline of soil fertility. This research aimed to assess whether and to what extent a newly introduced legume-based intercropping system is able to ameliorate the biological fertility status of an arable soil in a way that is still noticeable during the succeeding durum wheat cropping season in terms of changes in bacterial community structure, soil C and N pools, and crop yield. A field experiment was carried out under rainfed conditions in Southern Italy on a sandy clay loam soil cultivated with durum wheat following in the rotation a recently established grain legume (pea, faba bean)-barley intercropping. Soil chemical, biochemical and ecophysiological variables together with compositional shifts in the bacterial community structure by LH-PCR fingerprinting were determined at four sampling times during the durum wheat cropping season. Soil fertility was estimated by using a revised version of the biological fertility index. Results showed that even though the microbial biomass was significantly altered, the preceding legume intercrops stimulated C-related functional variables thus leading to an increased release of mineral N, which was larger in crop treatments succeeding pea-based than faba bean-based intercropping. The increased N made available in soil enabled the succeeding durum wheat to achieve an adequate grain yield with a reduced N-fertilizer use. Soil type and environmental conditions rather than crop treatments were major determinant of bacterial community structure. The biological fertility status was not varied, suggesting that in intensively managed rainfed areas long-term crop rotations with intercropped legumes are needed to consistently ameliorate it.

Keywords: intercropped legumes, residual soil N, soil C dynamics, biological fertility index, LH-PCR, succeeding crop

1. Introduction

Intercropping can be referred to as an ancient and traditional cropping system, but it has recently shown a serious potential to contribute to modern and sustainable agriculture. It represents the practice of cultivating two or more crops simultaneously in the same field for a considerable part of their life cycles (Vandermeer, 1989). A major agronomic success of intercropped systems relies on their complementary use of soil resources meaning that interspecific competition is weaker than intraspecific competition for growing factors (i.e. light, water and nutrients) (Willey, 1979). Intercropping including legumes was initially

practiced in tropical agriculture. However, legume-based intercropping is becoming increasingly appreciated also in areas other than the tropics because it is able to provide several agro-ecological services. These are more efficient means for use of environmental resources for plant growth due to a reduced competition for soil N (Hauggaard-Nielsen et al., 2003; Knudsen et al., 2004; Hauggaard-Nielsen and Jensen, 2005), an increased water and nutrient use efficiency (Hauggaard-Nielsen et al., 2009a), a greater yield stability and higher N concentration in cereal grain (Hauggaard-Nielsen et al., 2006, 2009b; Tosti and Guiducci, 2010), reduced nitrous oxide emissions from soil (Pappa et al., 2011), a better control of soil erosion (Inal et al., 2007), and an enhanced weed suppression and pest control (Liebman and Dyck, 1993; Corre-Hellou et al., 2011).

In a sustainable perspective, the agro-ecological role of intercropped legumes becomes of paramount importance for sustaining the productivity of cropping systems in low-input agricultural systems. This can be particularly appreciated in Mediterranean dry-prone areas where cereal (mainly barley and durum wheat) monoculture lead to a general depletion of soil N, which in turn affects grain yield, nutrient use efficiency and maintenance of soil fertility (Cossani et al., 2009). Moreover, severe climatic conditions exacerbate the decline of the organic pool, which represents a slow release nutrient source in arable soils. Thus given the large demand of crops for available N, deficiencies of N are a constant feature of Mediterranean agriculture and N fertilization is invariably required on an annual basis for non-legume crops, especially cereals (Ryan et al., 2008). Therefore, intercropping of legumes and cereals offers the opportunity to provide an input of biologically fixed N into the agro-ecosystem thus avoiding an excessive use of mineral N-fertilizer (Bedoussac and Justes, 2010). In previous field experiments recently introduced pea-barley intercropping systems resulted in increased yield stability as compared to grain legume monoculture (Hauggaard-Nielsen et al., 2009a). However, during the succeeding winter wheat cropping season contrasting responses were observed in terms of both grain yield and biomass production; while a general depletion of soil mineral N not dependent on the preceding crop was also found (Hauggaard-Nielsen et al., 2009a). A field experiment was therefore established with the intention of gaining an in-depth knowledge of soil fertility responses on an arable soil that had being cultivated with winter wheat for as long as one decade since the start of a crop rotation including a newly introduced legume-based intercropping system. In particular, this study focused on assessing if changes in functional and structural soil microbial community responses were taking place during the cultivation of durum wheat succeeding legumes-barley intercropping. Intercropped faba bean was introduced in the field experimental design to

evaluate its potential role as a substitute for grain pea, a crop commonly included in leguminous intercropping systems in semi-arid climates.

Changes in soil fertility in response to differing management systems are currently assessed by monitoring a wide range of soil physico-chemical and biological properties, which can also be combined in multiparametric indices (Bastida et al., 2008). One of these is the index of soil biological fertility (IBF), whose use has been suggested in Mediterranean agricultural soils, and provides an estimate of the fertility status of a soil as linked to C dynamics (Benedetti et al., 2006). However, critical revision of the index is still needed so as to avoid overestimation of the soil fertility status (Tortorella et al., 2013).

Given these premises, the specific purposes of the research were: (i) to assess any lasting effect of grain legumes-barley intercropping on C and N dynamics and on residual N made available in soil to the succeeding durum wheat for biomass production and grain yield; (ii) to validate the use of a modified class limits range for estimating the index of biological fertility (IBF); (iii) to verify whether compositional changes had occurred in the genetic structure of soil bacterial community as determined by the newly introduced intercropping system. To achieve these aims, a number of soil chemical (pH, $EC_{1:2}$, C_{org}, N_t, NH_4^+-N, NO_3^--N, exchangeable organic N, total soluble N), biochemical (MBC, MBN, R_{bas}, C_0, PMN) and microbial (MBC/C_{org}, *q*M, *q*CO_2, *q*CO_2/C_{org}) variables were measured during the durum wheat cropping season. The synthetic index of biological fertility of soil was calculated by considering the following C-related variables: C_{org}, R_{bas}, MBC, *q*CO_2, *q*M. Compositional shifts in the bacterial community structure due to the cropping systems were investigated by community fingerprinting based on length-heterogeneity PCR (LH-PCR) of PCR-amplified 16S rRNA gene fragments from soil-extracted bacterial DNA. The hypotheses assumed for this study were: in a rotation the recently established grain legume-based intercropping systems can stimulate soil C and N cycling in such a way that the additional N flow made available to the succeeding durum wheat is increased (H1); pea-based and faba bean-based intercropping systems provide differing effects on soil nutrient dynamics (H2); legume-based intercropping systems can effectively contribute to the restoration of the soil biological fertility status in rainfed agriculture (H3); together with the microbial activities also the bacterial community structure is markedly altered by the introduction of legume-based intercropping with cereals (H4).

2. Material and methods

2.1. Plant material and crop density

The plant species studied were grain pea (*Pisum sativum* L. cv Hardy), faba bean (*Vicia faba* L. cv Sikelia), six-row barley (*Hordeum vulgare* L. cv Aldebaran) and durum wheat (*Triticum turgidum* L. ssp. *durum* cv Virgilio). Sowing densities for full sole crops were 90 (grain pea), 40 (faba bean), 300 (six-row barley), and 350 (durum wheat) plants m^{-2}. Grain legumes and barley were intercropped (IC) in alternate rows (16 cm apart) to provide either a 100:50 additive design (100 and 50% of sole crop density for grain legume and barley respectively), or a 50:50 replacement design (both species at 50% of respective sole crop density).

2.2. Study site, experimental set-up and crop management

The field plots with legume-barley intercrops (IC) in a two-year rotation with durum wheat (DW) were established at the agricultural experimental center of the Regional Agency for Agriculture "ARSSA" (San Marco Argentano, Cosenza, Italy; 39°38'N, 16°13'E, 100 m above the sea level) on an arable soil that had been continuously cultivated with durum wheat for 10 years since the current experiment started in the 2010/2011 cropping season. The study area shows a typical Mediterranean climate, characterized by mild and rainy winters, relatively warm and dry summers and, generally, extended periods of sunshine throughout most of the year. Historical climatic data (averages over the 1995-2009 period) show that mean annual rainfall and air temperature for the area are, respectively, 709 mm and 16.1°C. The coldest month is February (mean temperature 3.0°C) and the hottest one is August (mean temperature 33.4°C). Soil thermal and moisture regimes are thermic and xeric, respectively. The soil evolves over the alluvial deposits from the nearest river Follone and is classified as Haplic Cambisol (Calcaric, Eutric) (IUSS Working Group WRB, 2006), or as Fluventic Haploxerept, coarse silty, mixed, thermic (Soil Survey Staff, 2010). Soil depth is generally greater than 120 cm and the available water holding capacity (AWC, available moisture between field capacity and wilting point) equals 20.0 cm. The soil is a sandy clay loam with the following characteristics: sand 55 ± 4%, silt 24 ± 5%, clay 21 ± 1%, bulk density 1.42 ± 0.14 g cm^{-3}; pH_{CaCl2} 7.67 ± 0.05, $EC_{1:2}$ 0.21 ± 0.03 dS m^{-1}, C_{org} 9.81 ± 0.25 g kg^{-1}, N_t 0.95 ± 0.04 g kg^{-1}, C/N 10.33 ± 0.51, C_{HA} 1.22 ± 0.25 g kg^{-1}, C_{FA} 0.51 ± 0.18 g kg^{-1}, CEC 26.2 ± 1.0 $cmol_{(+)}$ kg^{-1}, total $CaCO_3$ 18.0 ± 0.5 g kg^{-1}, active $CaCO_3$ 13.5 ± 0.4 g kg^{-1}, Olsen-P 12.5 ± 1.6 mg kg^{-1}, NH_4^+-N 17.0 ± 0.4 mg kg^{-1}, NO_3^--N 6.1 ± 0.5 mg kg^{-1}, DTPA-extractable Fe 7.6 ± 0.1 mg kg^{-1}, DTPA-extractable Mn 7.1 ± 0.1 mg kg^{-1}, DTPA-extractable Cu 1.6 ± 0.1 mg kg^{-1},

DTPA-extractable Zn 0.9 ± 0.1 mg kg^{-1}.

In the 2010/2011 cropping season, twenty-seven field plots (3 x 10 m each) were arranged in a randomized complete block design, with three replications, to compare the following nine IC treatments: grain legumes (pea, faba bean) and barley grown as either sole crop (P100, F100, B100, B100f) or intercropping in either an additive (P100B50, F100B50) or a replacement design (P50B50, F50B50). Field plots without plants were taken as a control treatment (Bare soil). In the following 2011/2012 cropping season the twenty-seven field plots (with the exception of the Bare soil) were cultivated with durum wheat (DW).

This paper takes into account the cropping season 2011/2012 during which DW field plots have been identified by the preceding IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f) plus the Bare control soil. DW following barley sole crop was further split in two distinct treatments: unfertilized (B100) and fertilized (B100f) field plots, which received an additional amount of 46 kg N ha^{-1} (as urea form) at the tillering stage (February 25, 2012). The seedling bed was prepared according to traditional tillage practices: ploughing (at a 30-cm depth) (November 7, 2011); disc harrowing combined with the addition of 200 kg ha^{-1} of a complex 18N - 46P_2O_5 chemical fertilizer (diammonium phosphate, DAP) supplying 36 kg N ha^{-1} and approx 40 kg P ha^{-1} (November 21, 2011); DW sowing with a precision line seeder (November 30, 2011). During the DW cropping season, the annual precipitation was 535 mm (from sowing to harvest), from which nearly as much as 50% occurred in February. No irrigation or chemical weed control was provided to the crop.

To determine aboveground biomass and grain yield, durum wheat plants were harvested at maturity (June 19, 2012) by destructive sampling in each plot from an area of 1 m^2. Sampled plants were separated into grains and straw (including stem, leaves and spikes), and then oven-dried at 65°C for 48 h before being weighed.

2.3. Soil sampling

Soil samples were collected at the following stages: pre-sowing (November 21, 2011), DW heading or ear emergence (May 2, 2012), DW harvest (June 19, 2012), and post-harvest (September 12, 2012). Three individual soil cores (approx 300 g each) consisting of bulk soil and soil particles loosely adhering to the root systems were taken at a 15-cm-depth from central rows of each plot so as to minimize any border effect, and then thoroughly mixed in a clean sampling bag to form a unique composite sample per field plot. A composite soil sample from unplanted field plots was considered as reference (Bare soil). Thus, three composite soil samples were collected per treatment and sampling time. Care was taken to keep the field

equipment sterile and prevent cross-contamination of soil samples during and after collection. A representative amount (approx 20 g) of each soil sample was immediately stored below 0°C in the field and then immediately processed for DNA extraction on return to the laboratory. Field moist samples were stored at 4°C before being partially air-dried, sieved at < 2-mm particle size, and promptly (within 24 h) processed for biochemical analysis, or stored again at 4°C for a maximum of seven days before chemical characterization.

2.4. Soil chemical and biochemical variables

Soil chemical properties (i.e. pH, $EC_{1:2}$, C_{org}, N_t,) were determined using the standard methods recommended by the Soil Science Society of America (Sparks, 1996). The microbial biomass C (MBC) and N (MBN) were determined according to the chloroform fumigation-extraction (CFE) procedure using a conversion factor of K_{EC} = 0.38 and K_{EN} = 0.54, respectively (Brookes and Joergensen, 2006). The soil basal respiration was determined as described by Öhlinger (1995). The cumulative CO_2-C evolved during a 28-day incubation period (readings after 1, 4, 7, 14, 21 and 28 days of incubation) was assumed as R_{bas}. The potentially mineralizable C pool (C_0) was estimated by fitting the 28-day cumulative CO_2-C readings to the first-order exponential function [$C_t = C_0 (1 - e^{-kt})$] (Eq. 1) (Riffaldi et al., 1996); C_t is the cumulative value of the mineralized C at time *t*, C_0 is the potentially mineralizable C pool, and *k* is an empirical constant describing the mineralization rate of the labile C pool. The best fitting of Eq. 1 to analytical data and estimates of the empirical parameters C_0 and *k* for each curve were carried out through non-linear regression analysis using the algorithm of Levenburg-Marquardt (TableCurve 2D v 5.01 software, SYSTAT software Inc., Erkrath, D). Non-linear regression was repeated several times in order to minimize the sum of the square of the deviation between predicted and experimental values below the threshold of 0.01% between two consecutive fits. The NH_4^+-N and NO_3^--N content in 2 M KCl soil extracts was determined colorimetrically by using a Flow Injection Analysis System (FIAS 400 PerkinElmer, Inc., CT, USA) equipped with an AS90 Autosampler (PerkinElmer) and linked to a UV/Vis spectrophotometer Lambda 25 (PerkinElmer). A gas diffusion-mixed indicator method was used for measuring ammonium content in the extracts, whereas nitrate content was determined after reduction to nitrite with copperized cadmium and reaction with sulfanilamide and N-(1-naphthyl)-ethylenediamine in HCl solution to form an azo-chromophore. The extractable organic N (EON) was calculated as the difference between the amount of total N (Kjeldahl method) and NH_4^+-N (FIAS method) occurring in 2 M KCl soil extracts. The potentially mineralizable N (PMN), resulting from net mineralization of the

active soil organic N pool during the 28-day incubation period for the R_{bas} determination was estimated as the cumulative soil inorganic-N released after 28 days *minus* the cumulative inorganic soil N at day 0 (Drinkwater et al., 1996). The following derived soil eco-physiological indices were then calculated: the microbial quotient (MBC/C_{org}), the metabolic quotient (qCO_2), the mineralization coefficient ($qM = R_{bas}/C_{org}$), and the qCO_2/C_{org} ratio (Dilly et al., 2001; Mocali et al., 2008). The synthetic index of biological fertility of soil (IBF) was calculated for all the treatments at the pre-sowing and harvest stage according to Benedetti et al. (2006). However, the following scheme was also used for rating the IBF into the five class fertility limits as proposed in the present paper: 6-10 (class I, alert), 11-15 (class II, low fertility), 16-20 (class III, average fertility), 21-25 (class IV, good fertility) and 26-30 (class V, high fertility).

2.5. Soil bacterial community analysis

Total bacterial community DNA was extracted from 2 g of freshly collected soil by using a direct extraction method (van Elsas et al., 1997) based on mechanical cell lysis with beads followed by sodium dodecylsulfate (SDS) treatment, phenol extraction and GeneClean® Spin Kit (QBiogene/MP Biomedicals, LLC, OH; cat. No. 1101-400) column filtration. Molecular community fingerprinting of soil bacterial community was carried out by length heterogeneity polymerase chain reaction (LH-PCR). PCR of purified soil extracts was carried out using the primer system fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 5'FAM-labelled PRUN518r (5'-ATTACCGCGGCTGCTGG-3') targeting the first third of the 16S rRNA gene sequence according to the protocol described by Mikkonen et al. (2011) based on Tiirola et al. (2003). The amplicons were separated according to their length polymorphism by polyacrylamide capillary electrophoresis (CE) using an ABI PRISM 310 Genetic Analyzer equipped with a 47-cm capillary and POP-6 Polymer (Applied Biosystems, Foster City, CA, USA). Briefly, the 15.00 µl CE samples were prepared by thoroughly mixing 3.00 µl of PCR products with 9.00 to 10.25 µl Hi-Di formamide and 1.75 to 3.00 µl of HEX-labeled products of known length (Tiirola et al., 2003) to give the final sample volume of 15 µl. CE running conditions were as reported by Mikkonen et al. (2011).

2.6. Statistical analysis

Soil chemical and biochemical data, reported as mean values ($n = 3$), were expressed on a dry weight (dw) basis (105°C, 24 h), and first tested for deviation from normality (Kolmogorov-Smirnov test) and homogeneity of within-group variances (Levene's test). After running a two-way analysis of variance (ANOVA, time x preceding crop) to check any significant effect

of time, preceding crop and their interaction on the variability of the data (the block effect in the experimental design was found to be not significant at P <0.05), a multiple pairwise comparison of means was done by Tukey's HSD (honestly significant difference) test at P <0.05 level of significance. Crop data (total aboveground biomass and grain yield) shown in Table 3 were analyzed by a multiple pairwise comparison of means (Tukey's HSD test at P <0.05). Principal component analysis (PCA) with no rotation was also used to statistically process the soil chemical and biochemical dataset. Statistical analyses were run using the Systat 11.0 software (SYSTAT Software Inc.). Graphs were drawn by using the SigmaPlot 10.0 software (SYSTAT Software Inc.). LH-PCR community fingerprinting data were analyzed using the BioNumerics v. 6.6 (Applied Maths, Kortrijk, B) as described by Mikkonen et al. (2011). Dendrograms of hierarchical classification were generated by cluster analysis using the unweighed pair-group method with arithmetic averages (UPGMA) based on Dice similarity coefficient.

3. Results

3.1. Grain yield and biomass production

Crop data evidenced that grain yield was higher in DW after legume-based IC combinations than in DW after barley, except where additional fertilizer-N was supplied (Table 1). Moreover, both the grain yield and biomass production were also generally higher where durum wheat succeeded a pea-based rather than a faba bean-based intercropping (Table 1). In detail, the highest and the lowest biomass production were observed in the field plot following the P100 and the B100 treatments, respectively. Durum wheat grain yield showed a similar behavior: when supplied with an additional amount of fertilizer-N the yield in the barley-succeeding treatment (B100f) was comparable to that obtained in succeeding legume-based intercropping combinations (Table 1).

3.2. Soil pH and EC

During the 2011/2012 cropping season, either the soil pH or EC were significantly influenced only by the time factor (Fig. S1). In particular, the former showed a decrease in all treatments (from average 7.64 to 7.36) between the third (Jun 2012) and the fourth (Sept 2012) sampling. Whereas the latter fluctuated markedly across time: the lowest (average 0.15 dS m^{-1}) and the highest (average 0.18 dS m^{-1}) values were observed at the second (May 2012) and fourth (Sept 2012) sampling time, respectively.

3.3. Soil C pools

The two-way ANOVA showed that time was the main factor that affected the variability of all the considered chemical and biochemical variables (Figs. 1, 2 and S2); whereas, the preceding crop and the time x preceding crop interaction exerted a significant effect on a limited number of soil C variables (C_{org}, R_{bas}, C_0 and *q*M).

Soil organic carbon (C_{org}) ranged between 9.14 and 10.73 mg g^{-1} and the two-way ANOVA showed that both the time and the preceding crop had a significant influence on the variability of the data (Fig. 1). Besides clear time-dependent fluctuations, a noticeable difference among treatments in the soil C_{org} content was observed over the growing season, especially at the harvest stage. In particular, the lowest values (< 10 mg g^{-1}) were recorded in both B100 and B100f treatments; whereas an average C_{org} content up to 10.14 and 10.28 mg g^{-1}, respectively, was found in the DW field plots following in the rotation the pea-based (P100, P100B50, P50B50) or faba bean-based (F100, F100B50, F50B50) IC treatments (Fig. 1). Only a slight variation of soil C_{org} was recorded in the unplanted control soil.

It is worth noting that all the three following C-related biochemical variables (i.e. R_{bas}, C_0 and *q*M) were statistically influenced by time, preceding crop and their interaction (Fig. 2). Even though R_{bas} readings were not statistically different at the pre-sowing stage (values ranging between 235.5 and 257.5 µg CO_2-C evolved g^{-1} 28 d^{-1}), at the second sampling a noticeable difference was found among treatments. Precisely, R_{bas} increased markedly in B100, B100f and in the Bare soil (up to approximately 269 µg CO_2-C g^{-1} 28 d^{-1}); whereas a significant decline was observed in both faba bean-based (down to 230 µg CO_2-C g^{-1} 28 d^{-1}) and pea-based IC (down to 203 CO_2-C g^{-1} 28 d^{-1}) (Fig. 2). R_{bas} declined significantly in all treatments at the harvest stage. In late summer, a considerable discrepancy was observed among treatments: R_{bas} went up sharply in most treatments except in the control soil and in P100, where it showed a marked decline. Noticeably, the highest R_{bas} values were found in faba bean-based IC systems. The potentially mineralizable C (C_0) showed the same behavior observed for the R_{bas}: a clear decreasing pattern from sowing to the harvest stage and a late raise during the summer (Fig. 2). The *q*M showed a declining trend from the pre-sowing to the harvest stage that was followed by a slight increase at the late summer sampling (Fig. 2). Moreover, there were also considerable discrepancies among treatments over the growing season. In the DW field plots following faba bean-based IC (namely F100B50 and F50B50) the *q*M increased over time and higher values were observed at the final stage (23.4 and 23.3 µg CO_2-C mg^{-1} C_{org}, respectively). On the contrary, in barley-succeeding plots the *q*M showed marked seasonal variations and finally decreased to values lower than the initial ones. A more marked decline over time was found in both pea-based IC (e.g. 15.5 µg CO_2-C mg^{-1} C_{org} in

P100) and unplanted control soil (15.4 µg CO_2-C mg^{-1} C_{org}) (Fig. 2).

The two-way ANOVA showed that time was the only factor affecting the variability of MBC and MBC/C_{org}, with no significant differences among treatments (Fig. S2). Precisely, MBC generally decreased from sowing to the ear stage; then, a slight increase was observed at the harvest and post-harvest stages. Noticeably, field plots following faba bean-based IC showed a somewhat contrasting behavior: in F100B50 MBC firstly increased from 147.37 µg MBC g^{-1} (sowing) to 231.58 µg MBC g^{-1} (harvest), and then decreased to 113.16 µg g^{-1} (late summer). Conversely, in F50B50 the MBC remained unchanged at around 134 µg g^{-1} from sowing to harvest, and then increased consistently until reaching the highest value (242.98 µg g^{-1}) at the last sampling. A similar trend was observed in the microbial quotient: it declined slightly at initial stages and then increased in late samplings (Fig. S2). Major time-dependent variations were observed in F100B50 where the MBC/C_{org} increased markedly from the pre-sowing to the harvest stage (+63% compared to the initial value of 14.2 µg MBC mg^{-1} C_{org}) and then sharply declined at the last sampling (i.e. from 22.81 to 11.31 µg MBC mg^{-1} C_{org}). In the bare soil, the microbial quotient fluctuated over time and the variations were primarily due to the decline in MBC than to changes in C_{org} (Fig. S2).

Either the qCO_2 or the qCO_2/C_{org} ratio increased considerably between the first and the second sampling time with no significant differences among treatments (Fig. S2). In brief, these two variables declined first sharply and then slightly from the earing to the post-harvest stage in all theses but the F100B50, where they markedly increased until reaching the highest values across the whole season (0.3 µg CO_2-C $µg^{-1}$ MBC d^{-1}) (Fig. S2).

3.4. Soil N pools

The two-way ANOVA showed that the total soil nitrogen content (N_t) was significantly influenced ($P < 0.001$) by either time or the preceding crop factors (Fig. 1). N_t declined constantly in all treatments across time, except in the bare control soil where it remained constant over time, and the lowest soil N_t content was found (< 1 mg g^{-1}) at three out of four samplings. Furthermore, considerable discrepancy was found among the cultivated treatments during the cropping season. That is, the N_t decline was more evident in the DW field plots following pea-based (P100, P100B50, P50B50) than those following faba bean-based treatments (F100, F100B50, F50B50) (Fig. 1). It is also worth noting that the N_t decreased more remarkably in the two barley-succeeding treatments where a sharp drop was observed: precisely, from 1.3 to 1.0 mg g^{-1} in B100f, and from 1.2 to 0.95 mg g^{-1} in B100 (Fig. 1).

The two-way ANOVA also showed that the two main factors (time and preceding crop)

and their interaction statistically influenced either inorganic-N form (namely NH_4^+-N and NO_3^--N) (Fig. 3). The NH_4^+-N content slightly varied (values around 21 µg g^{-1}) among treatments and over time, with the exception of the harvest stage, where significantly higher and contrasting values were observed. In detail, the NH_4^+-N content was on average by 7% larger in those DW field plots following the three pea-based and two faba bean-based (F100, F100B50) IC together with B100. An even larger increase was found in the F50B50 treatment (from 20.2 to 23.1 µg NH_4^+-N g^{-1}), in the B100f treatment (from 20.1 to 24.8 µg NH_4^+-N g^{-1}) and, above all, in the unplanted control soil (from 20.0 to 25.8 µg NH_4^+-N g^{-1}) (Fig. 3). Time affected considerably the dynamics of nitrate content in soil during the cropping season. The NO_3^--N content dropped sharply between the pre-sowing and the heading stage, remained constant until the harvest stage and then rose dramatically before the late summer sampling, when it reached final values even higher than those at the pre-sowing stage (Fig. 3). However, besides time-dependent changes there was a considerable discrepancy among treatments across the entire experimental period. In particular, large soil nitrate content was assessed in the DW field plots following legume-based IC and the most marked differences between legume-based IC and the other treatments were found at the beginning of the 2011/2012 cropping season. The lowest soil nitrate content was in most cases observed in the field plots kept under continuous cereal cultivation (i.e. durum wheat after barley) (Fig. 3). Even though the bare soil showed initial values similar to the other treatments (6.9 *vs* 7.0 µg NO_3^--N g^{-1} on average), the NO_3^--N content went up considerably over time until it reached a final value nearly twice as it was at the beginning of the cropping season.

Both time and its interaction with the preceding crop significantly affected the extractable organic N (EON) (Fig. 4). The EON content decreased between the pre-sowing stage and the second sampling, and then increased slightly over the last two sampling stages. In the control soil, final EON values were similar to the initial ones (72.8 *vs* 76.7 µg N g^{-1}). On the other hand, the total soluble N (TSN) was statistically influenced by both the preceding crop and the sampling time, but not by their interaction (Fig. 4). The TSN showed time-dependent variations similar to those observed in the EON. A large TSN content was generally evidenced in the DW field plot following the legume-based IC (Fig. 4). Once again, the bare soil treatment showed similarity between initial and final values (99.6 *vs* 112. 8 µg N g^{-1}, respectively).

Finally, significant ($P < 0.001$) time-dependent variations were observed also in the PMN and MBN data (Fig. S3). Precisely, from the first to the third sampling MBN declined in all theses (-29% on average), and then it increased again during the post-harvest period.

Whereas, PMN data markedly fluctuated across the cropping season, with no noticeable differences among treatments.

3.5. Multivariate analysis of soil chemical and biochemical variables

Principal component analysis (PCA) of soil chemical and biochemical variables monitored during the 2011/2012 cropping season was able to factorize the variation of the dataset into six most significant components (Factor 1, 2, 3, 4, 5 and 6) according to the "eigenvalue > 1.0" criterion). Taken all together these six principal components explained approximately 84.2% of the total variance of the dataset (Table 2). In detail, PC1 accounted for 29.45% of the total variance and was primarily affected by the following soil C-related biochemical variables: the functional variables qCO_2, the qCO_2/C_{org} ratio, qM and R_{bas} that were inversely correlated to the structural variables MBC and MBC/C_{org}). On the other hand, PC2 accounted for 19.59% of the total variance and was weighed by both C- and N-related functional variables, namely R_{bas}, C_0, TSN, EON that were all inversely related to NH_4^+-N. Moreover, pH and PMN were the main variables that were represented in PC3 (12.14% of the total variance). Indeed, PC4 was not markedly weighed by any soil variable. Finally, C_{org} had a noticeable weight onto PC5 (7.22%), and PMN onto PC6 (6.27%). Three N-related variables (NO_3^--N, MBN and N_t,) together with EC had no significant weight on any selected principal component.

Since the first two PC taken together accounted for as much as half of the total explained variance (approx 49%), we focused on these two to represent loadings and scores in a PCA ordination biplot. The DW treatments following the legume-based IC were well separated from each other (especially pea-based treatments along the PC1) and they could be broadly clustered in two main groups along the PC2 axis (Fig. 5). In addition, the two barley-succeeding DW treatments were also well discriminated from each other, but the B100f shifted towards the faba bean-based treatments. The unplanted control soil was apart from the cultivated treatments, with the exception of the P100 thesis (Fig. 5).

3.6. Index of biological fertility of soil (IBF)

According to the fertility class rating system proposed by Benedetti et al. (2006) at the pre-sowing stage the IBF ranked within the class III (average fertility, score ranging from 14 to 15) in all treatments (Table 3). At the harvest stage the IBF declined in most cases, with the exception of the F100 (constant value) and F100B50 (minor increase) (Table 3). A change in the soil fertility class was found in the two barley-succeeding DW treatments (namely B100 and B100f), where the IBF declined to as low as 12. This value represents the soil fertility

class II, which indicates a low biological fertility status and hence a condition of vulnerability of soil resources. On the contrary, according to our newly proposed class rating system (6-10, class I, alert; 11-15, class II, low fertility; 16-20, class III, average fertility; 21-25, class IV, good fertility; 26-30, class V, high fertility), the IBF always ranked within the fertility class II (low biological fertility, warning condition), in spite of the crop treatment or the sampling time (Table 3).

3.7. Soil bacterial community structure

LH-PCR averaged profiles of soil samples taken at pre-sowing stage showed that the soil bacterial community structure was still affected by the preceding crops, regardless of the preceding IC treatment (Fig. 6A). This finding becomes clear in the similarity dendrogram shown in Fig. 7A. The unplanted control soil is well separated from the cultivated treatments at 80% similarity level. Noticeably, at the end of the DW cropping season, the distinction between the bare soil and the other treatments was still noticeable (Fig. 6B), and a slightly increased divergence was noticed (level of similarity of approximately 79%, Fig. 7B), mainly due to the 16S rRNA gene-coding fragments of the control soil having a length between 500 and 545 bp (Fig. 6B). Furthermore, legume-based IC treatments clustered all together in a broad group that could be discriminated from monoculture cereals at ~83% similarity (Fig. 7B).

4. Discussion

4.1. Soil pH and EC

Apart from noticeable time-dependent fluctuations neither pH nor EC were affected by the differing cropping systems. This finding cannot be considered particularly surprising as either variable originates as a result of the underlying soil formation process and any their variation is therefore expected to arise over the very long term.

4.2. Soil C and N pools

The lasting effect of a combination of a recently established grain legume-barley intercropping system on the fertility status of a sandy clay loam soil during the succeeding cultivation of durum wheat was clearly appreciable. This finding was especially noticeable in terms of significantly higher total organic C and N pools, which were also functionally linked to C dynamics and soluble N forms release. The increase in soil organic resources is in line with what had already been observed at the harvest stage in pea- and faba bean-cultivated plots before the 2011/2012 growing season started (Tortorella et al., 2013); and it further

confirms that legume-based intercropping may create conditions which are conducive to the maintenance of organic pool in soil. Nevertheless, restoration of soil organic resources becomes critical in Mediterranean low rainfall areas where total organic C is generally below the critical threshold of 2% (Zdruli et al., 2004), and continuous cereal (i.e. barley-durum wheat) cultivation exacerbate the loss of soil organic matter (Ryan et al., 2008) and leads to a general depletion of available soil N (Cossani et al., 2009). It is known that legume plants are able to release into the soil a large amount of easily mineralizable organic substrates, namely root exudates and rhizodeposits (Jensen, 1996). These substrates are particularly rich in N-containing organic forms that represents from 14 to 39% of total plant N derived from biologically fixed N_2 (Jensen et al., 2010). The N-enriched compounds entering the soil are prone to mineralization and stimulate the microbial activity (Hauggaard-Nielsen et al., 2008). The overall result is an increased release of mineral N forms and an altered N pool dynamics (Song et al., 2007a). This was especially true for our intercropping-succeeding treatments: an additional amount of soil nitrate (estimated as high as approximately +24 and +10 kg ha^{-1} in pea-based and faba bean-based combinations, respectively) as compared to barley-succeeding treatments was found at the pre-sowing stage. These readings are consistent with what previously observed (Jensen, 1996; Hauggaard-Nielsen et al., 2009b), but may also be contrasting with the additional amount of N deposited belowground (up to 100 kg N ha^{-1}) found under faba bean cultivation (Jensen et al., 2010). Nevertheless, any discrepancy observed in the two legumes ability to increase the soil C and N sink could be explained by the different quality and quantity of easily mineralizable substrates (rhizodeposits and crop residues) released from the grain legumes root systems (Mayer et al., 2004). It is also known that long-term cropping history together with specific local conditions can exert a great influence on residue mineralization rates and thus on soil N dynamics (Hauggaard-Nielsen et al., 2009b). The increased N availability occurring in soil at the beginning of the growing season can therefore explain the crop yield and aboveground biomass production found in the field plots cultivated with the intercropping-succeeding durum wheat: they were significantly higher than those obtained in barley-succeeding plots, but only when no additional fertilizer-N was supplied. In fact, there was a clear disadvantage in term of both grain yield and biomass production in monoculture cereal. This adverse effect was mitigated, at least partially, by the preceding legume-based intercropping, especially pea-based combination. Even though decaying legume residues have been show to increase soil P availability and P uptake to the succeeding wheat (Hasbullah et al., 2011) these aspects were not considered here and the study of soil C and N cycling as related to the assessment of the biological fertility status have

represented the main focus of the work.

Taken together, these results suggest that the increase in easily mineralizable N-containing rhizodeposits entering the soil from grain legume roots could have stimulated the mineralization of crop residues as also indicated by the increased C dynamics and related functional variables, i.e. R_{bas}, *q*M and qCO_2 and qCO_2/C_{org}. Indeed, these variables were not statistically related to the microbial biomass N, which remained unaffected. On the other hand, they showed marked time-dependent variations (according to ANOVA) and were inversely related to the MBC (according to PCA). This finding is not in line with the MBC increase observed in intercropped maize-soyabean (Oelbermann and Echarte (2011) or wheat-faba bean (Song et al., 2007b). It is worth saying that these two papers refer to a field research carried out under pedoclimatic and management conditions (i.e. Pampa, 860 mm rainfall, C_{org} 1.6%; northwestern China, irrigation, C_{org} 2.0%) quite contrasting with those of our experimental site (535 mm rainfall, C_{org} <1.0%). Therefore, it is plausible that in our study preceding legume-based intercropping was primarily able to stimulate the microbial activity rather than microbial growth, and this effect lasted over the following cropping season. If this happens, then all C-related functional variables rise considerably.

To summarize, over the short term decaying legume crop residues lead to an increase of the available N pool in soil. However, in arable soils where the soil fertility level is far from being adequate, or if organic amendments or crop residues are not continuously being incorporated into soil this effect contributes only partially to the N supply for the succeeding cereal and might not persist over a longer period. Thereupon, H1 is only partially confirmed and the residual N made available in soil from decaying legume residues can benefit the succeeding durum wheat (and save fertilizer-N use) provided prolonged intercropping is adopted. Moreover, both pea-barley combinations (either additive or replacement design) enabled a better yield performance of the succeeding durum wheat than faba bean-based intercropping due to a larger release of mineral N (H2 uneven action of the intercrops). In brief, in continuous cereal cultivation the exploitation of available soil N could properly be faced by using rotational approaches with intercropped legumes, provided they are grown for several years before any preceding crop effect can become clearly appreciable, especially under low fertility level.

4.3. Index of biological fertility of soil

Our newly proposed class rating system provided estimates of the biological fertility status that were lower than otherwise assessed, but more consistent with what observed in the field

plots. The newly proposed narrower class limits are able to discriminate soil fertility conditions more accurately. In fact, according to the original criterion for ranking the IBF within different fertility classes (Benedetti et al., 2006), the fertility class I (limits from zero to six) actually represents a single case instead of a wide range of low soil fertility conditions. As well as this, only the upper limit of six (which also represents the lowest limit of class II) can be achieved in practice and shifting values towards the upper class limits leads to a general overestimation of the IBF over the entire fertility class range. However, that was not the case when applying a modified class rating system, thus confirming the need for its revision. This would also help identify at an earlier stage soil conditions before they become critical. It can be observed that continuous cereal cultivation (i.e., durum wheat succeeding barley) negatively affected the fertility status of the soil. However, this decline was not significantly mitigated when including a preceding intercropped combination. This brings to the conclusion that due to the general low fertility level of the sandy clay loam soil any decisive improvement in the fertility status cannot be expected to happen in the short-term nor it can be easily achieved unless intercropped systems are being adopted for several years. Thus, H3 cannot be confirmed in our case study unless repeated observations over several consecutive cropping seasons are carried out.

4.4. Soil bacterial community structure

Bacterial community fingerprinting by LH-PCR clearly showed that the cropping treatment had only a minor role on shaping the bacterial community composition. Any crop-induced effect on soil bacterial community seemed fairly transient in soil and did not extend over the following cropping season. In fact, at the pre-sowing stage the residual effect as due to the preceding legume-based intercropping system was still noticeable. Whereas, at the harvest stage of the succeeding durum wheat any appreciable difference among treatments was greatly reduced. Noticeable differences on bacterial community composition as due to intercropped legumes have been reported elsewhere, even though different community fingerprinting methodologies were used, such as phospholipid fatty acid (PLFA) profiling (Chen et al., 2008; Li et al. 2010), PCR-DGGE community fingerprinting (Song et al., 2007a, b), and terminal restriction fragment length polymorphism (t-RFLP) (Sun et al., 2009). It must be also noted that LH-PCR fingerprinting describes the molecular composition of a soil bacterial community and represents the mix of taxa occurring in the soil extract. Yet it cannot be used to identify specific taxa (Oros-Sichler et al., 2007). This makes our finding consisting with what reported by Sun et al. (2009) and Zhang et al. (2010), who concluded that only a

slight effect of intercropping on the molecular structure could be observed when the soil bacterial community was investigated at phylum level. This also brings to the conclusion that under the present cropping conditions dominated by the severe climatic constraints typical of Mediterranean agro-ecosystems (i.e. soil dryness for prolonged periods, paucity of soil organic matter, accelerate pedogenesis), the bacterial community structure was primarily determined by specific soil physical and chemical properties rather than by soil management or crop effect (Cookson et al., 2006).

In brief, no compositional changes in the genetic structure of soil bacterial community were observed in response to the newly introduced intercropping system. Therefore, H4 was not confirmed. However, it cannot be excluded that changes in less abundant and species-specific bacterial groups could have remained undetected. This constitutes the main aim of ongoing research.

4.5 Conclusions

Our findings demonstrate that under rainfed crop conditions a recently established legume-barley intercropping was able to significantly affect either the soil C and N pools or the microbial activity in a way that it was still noticeable during the following cropping season with durum wheat. This was especially true for total C and N pools, which were increased soon after the legume-based intercropping season. The temperature-moisture regimes typical of a Mediterranean dryland area stimulate the mineralization process of plant-derived C-substrates, especially N-enriched compounds entering the soil from decaying legume root tissues. The result is therefore an increased initial supply of soluble N forms (especially nitrate) that meets the N-demand of the following durum wheat and allows a comparable grain yield with a reduced N-fertilizer use. This effect might not persist over a longer period, and any residual N made available in soil from decaying legume residues can benefit the succeeding durum wheat (and save fertilizer-N use) provided prolonged intercropping is adopted. It is also interesting to note how the largest release of available soil N was observed following pea-based, proving that in a crop rotation pea-based combination (either in additive or replacement design) are more beneficial than faba bean-based combinations to the succeeding cereal. Moreover, no consistent relationship was found between the bacterial community structure and changes of functional properties in soil; thus confirming the resilience of the soil bacterial community, at least at higher taxa level, to the newly introduced cropping systems. Revised class limits rating the five fertility levels provide a more reliable estimate of the IBF and a more accurate distinction among soil fertility conditions. To sum up,

the results indicate that in intensively managed rainfed areas any decisive improvement in the soil fertility status cannot be rapidly or easily achieved, unless crop rotations with intercropped legumes are adopted for several years.

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Table 1. Grain yield and total aboveground biomass (mean ± SD, *n=3*) at the harvest stage of durum wheat cultivated in field plots following IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50 and B100f as in § 2.2) in a two-year crop rotation. Crop data are from the 2011/2012 cropping season. Different letters in a column denote significant differences (Tukey's HSD test, $P < 0.05$).

Crop treatment	Grain yield	Aboveground biomass
	(t dw ha^{-1})	
P100	4.3 ± 0.3 ^{a}	8.8 ± 0.7 ^{a}
P100B50	3.8 ± 0.4 ^{ab}	7.6 ± 1.0 ^{ab}
P50B50	3.4 ± 0.3 ^{ab}	7.1 ± 0.7 ^{ab}
B100	2.3 ± 0.2 ^{c}	4.9 ± 0.6 ^{c}
F100	3.6 ± 0.5 ^{ab}	7.7 ± 1.3 ^{ab}
F100B50	3.2 ± 0.4 ^{b}	6.5 ± 0.7 ^{bc}
F50B50	3.0 ± 0.5 ^{bc}	6.1 ± 1.0 ^{bc}
B100f	3.3 ± 0.4 ^{b}	6.5 ± 1.0 ^{bc}

Table 2. Principal component analysis (PCA) of 17 chemical and biochemical soil variables measured in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Samples from the four sampling times during the 2011/2012 cropping season were considered all together in the PCA analysis. PC loading variables (values ≥ |0.60| are in bold) and percent of total variance explained by the first six factors (eigenvalue > 1) are reported.

Soil variable	PC1	PC2	PC3	PC4	PC5	PC6
qCO_2	**-0.92**	0.01	0.18	0.22	-0.07	0.04
qCO_2/C_{org}	**-0.91**	-0.02	0.20	0.25	0.01	-0.03
MBC	**0.83**	0.21	0.07	-0.47	0.08	0.03
MBC/C_{org}	**0.80**	0.18	0.05	-0.49	0.20	-0.05
*q*M	**-0.68**	0.51	0.24	-0.30	0.29	-0.01
R_{bas}	**-0.63**	**0.60**	0.24	-0.31	0.06	0.19
NO_3^--N	0.52	0.50	0.37	0.38	-0.07	0.12
TSN	0.20	**0.71**	-0.41	0.45	0.12	-0.15
C_0	-0.47	**0.69**	0.08	-0.28	0.15	0.23
EON	0.09	**0.68**	-0.51	0.37	0.10	-0.21
NH_4^+-N	0.07	**-0.64**	-0.03	0.22	0.49	0.22
MBN	0.26	0.59	0.14	0.08	0.17	-0.08
pH	-0.50	-0.14	**-0.63**	-0.23	0.03	0.23
PMN	-0.12	-0.02	**0.61**	-0.10	-0.33	**-0.60**
N_t	-0.09	0.29	-0.53	-0.18	-0.41	-0.15
C_{org}	0.23	0.22	0.04	0.02	**-0.69**	0.55
EC	0.45	0.16	0.48	0.38	0.10	0.31
Variance explained (%)	*29.45*	*19.59*	*12.14*	*9.57*	*7.22*	*6.27*

Table 3. Index of biological fertility of soil and fertility classes in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at the pre-sowing and harvest stage during the 2011/2012 cropping season.

	Sampling time					
	Nov 2011			Jun 2012		
Treatment	IBF	Class [$]	Class [§]	IBF	Class [$]	Class [§]
P100	15	III	II	14	III	II
P100B50	15	III	II	14	III	II
P50B50	14	III	II	13	III	II
B100	14	III	II	12	II	II
F100	14	III	II	14	III	II
F100B50	14	III	II	15	III	II
F50B50	14	III	II	13	III	II
B100f	14	III	II	12	II	II
Bare soil	15	III	II	14	III	II

[$] According to Benedetti et al. (2006).
[§] According to the newly proposed fertility class rating system (as in § 2.4).

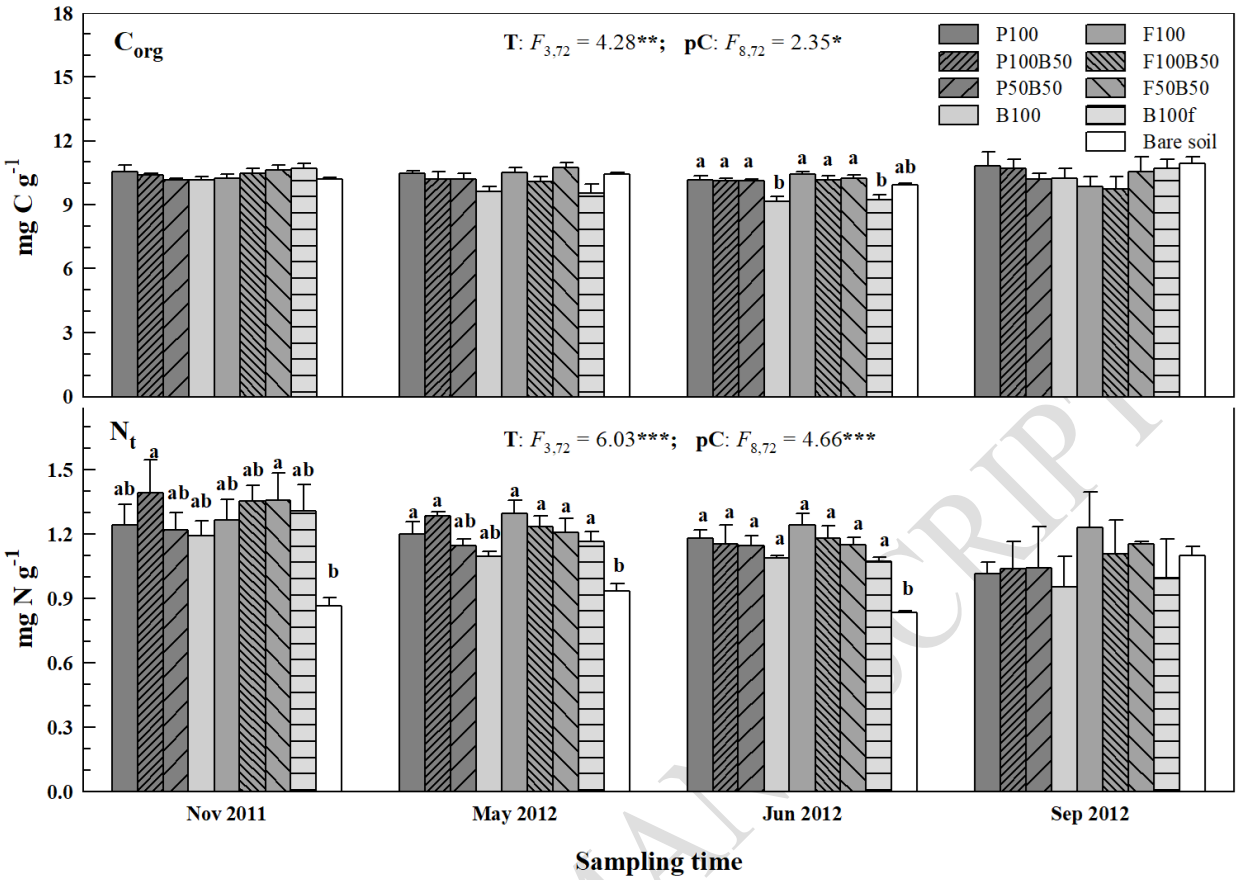

Fig. 1. Changes in total soil organic C (C_{org}) and N (N_t) (mean ± SD, *n=3*) in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at four stages (pre-sowing, ear emergence, harvest and post-harvest) during the 2011/2012 cropping season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $P < 0.05$). Significant effects due to time (T), preceding crop (pC) and their interaction are presented as *F*-values and level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) estimated by a two-way ANOVA (time x preceding crop).

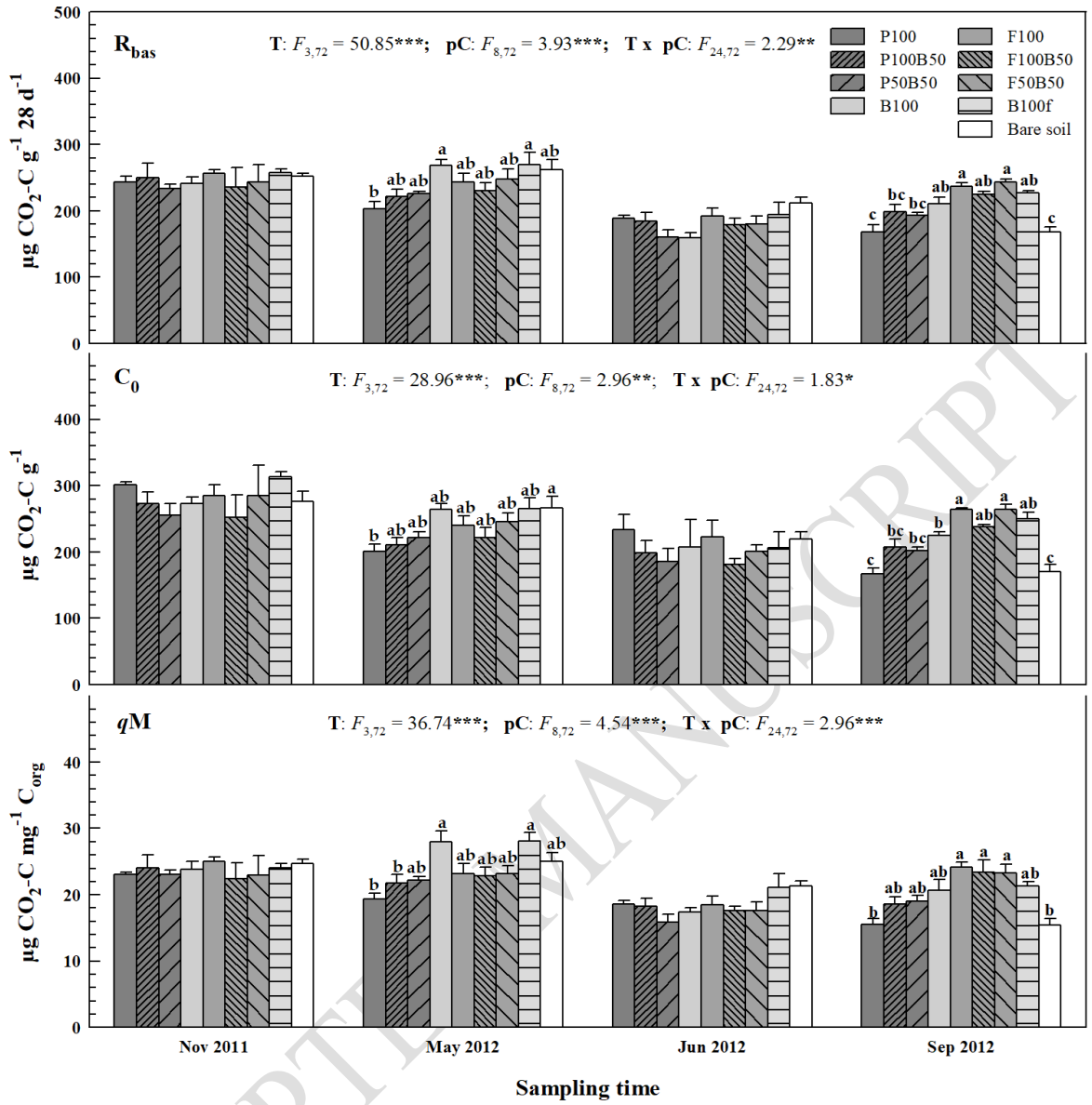

Fig. 2. Changes in soil basal respiration (R_{bas}), potentially mineralizable C (C_0) and mineralization coefficient (*q*M) (mean ± SD, *n=3*) in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at four stages (pre-sowing, ear emergence, harvest and post-harvest) during the 2011/2012 cropping season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $P < 0.05$). Significant effects due to time (T), preceding crop (pC) and their interaction are presented as *F*-values and level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) estimated by a two-way ANOVA (time x preceding crop).

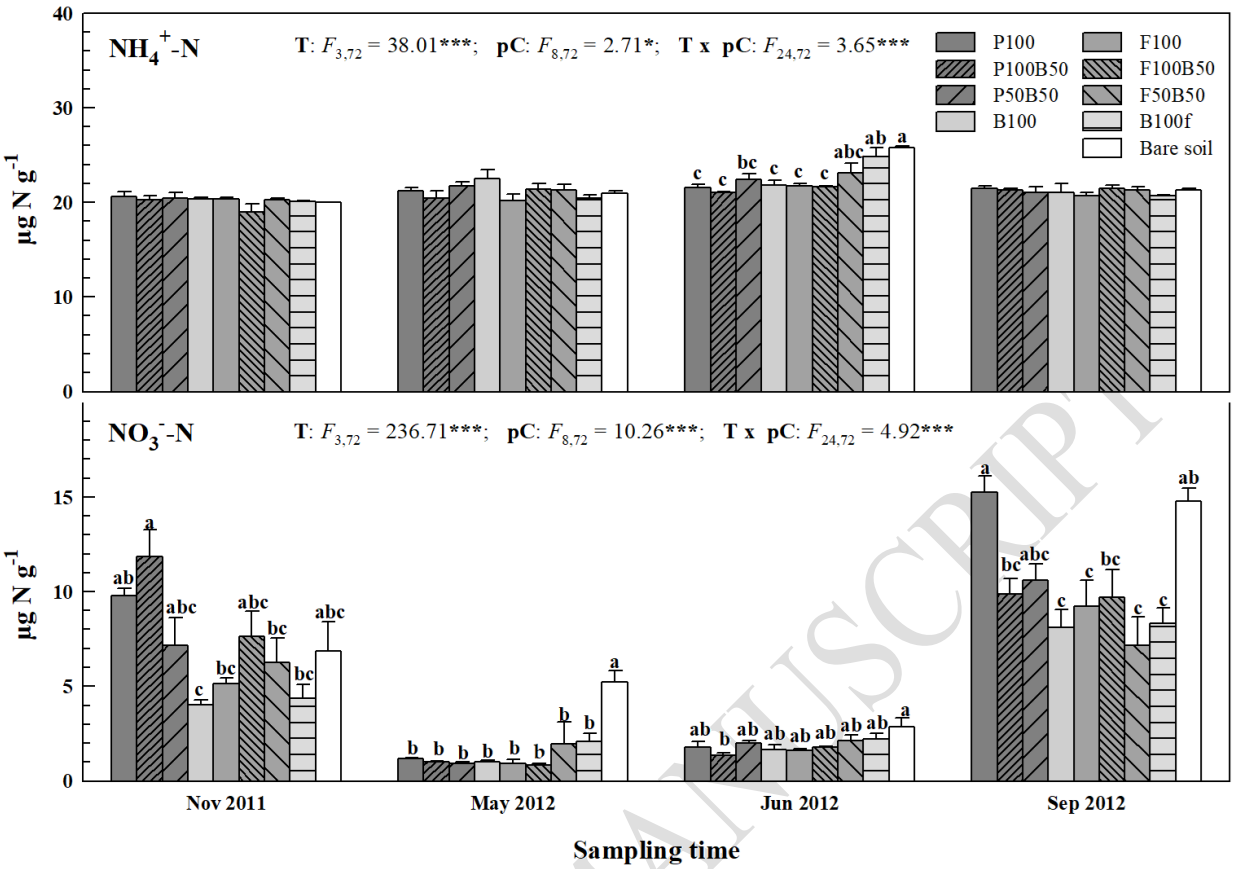

Fig. 3. Changes in soil ammonia N (NH_4^+-N) and nitrate N (NO_3^--N), extractable organic N (EON) and total soluble N (TSN) (mean ± SD, *n=3*) in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at four stages (pre-sowing, ear emergence, harvest and post-harvest) during the 2011/2012 cropping season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $P < 0.05$). Significant effects due to time (T), preceding crop (pC) and their interaction are presented as *F*-values and level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) estimated by a two-way ANOVA (time x preceding crop).

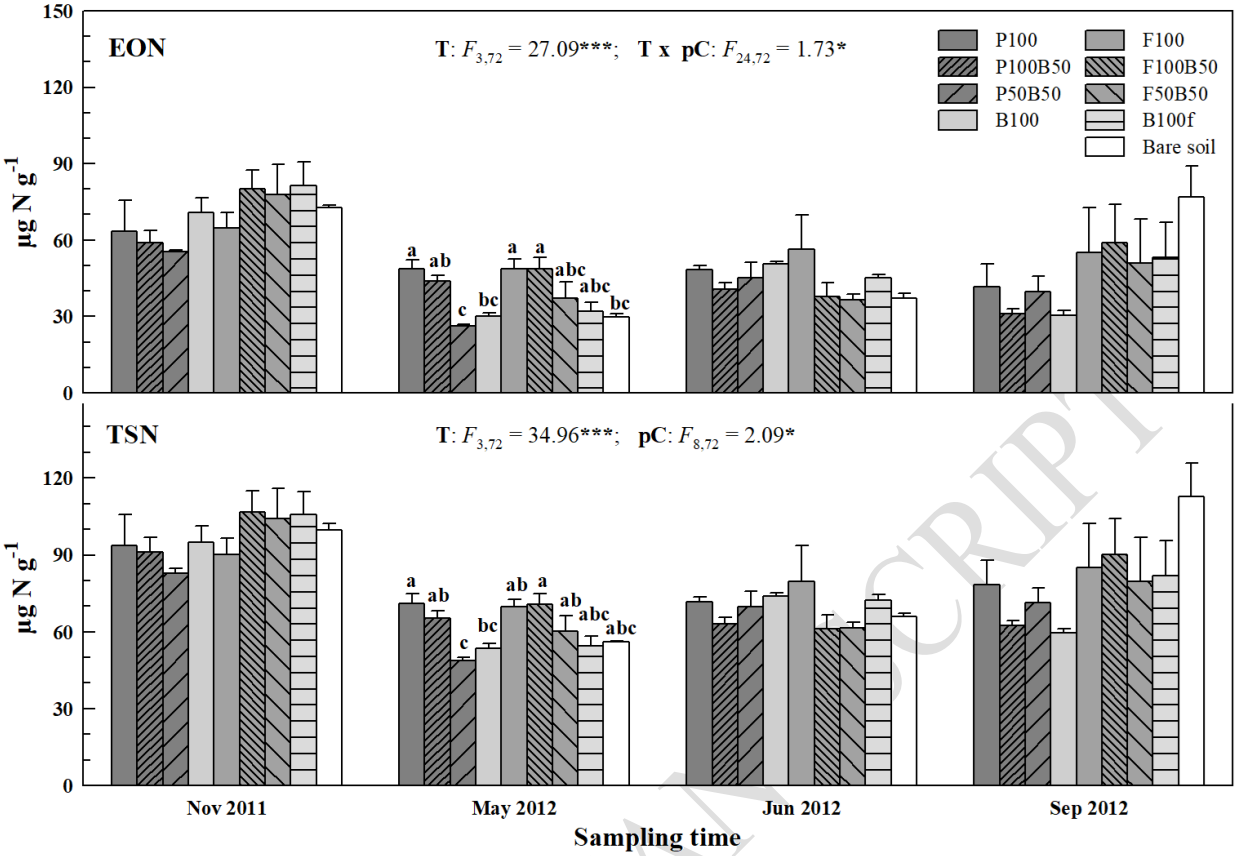

Fig. 4. Changes in soil extractable organic N (EON) and total soluble N (TSN) (mean ± SD, *n=3*) in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at four stages (pre-sowing, ear emergence, harvest and post-harvest) during the 2011/2012 cropping season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $P < 0.05$). Significant effects due to time (T), preceding crop (pC) and their interaction are presented as *F*-values and level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) estimated by a two-way ANOVA (time x preceding crop).

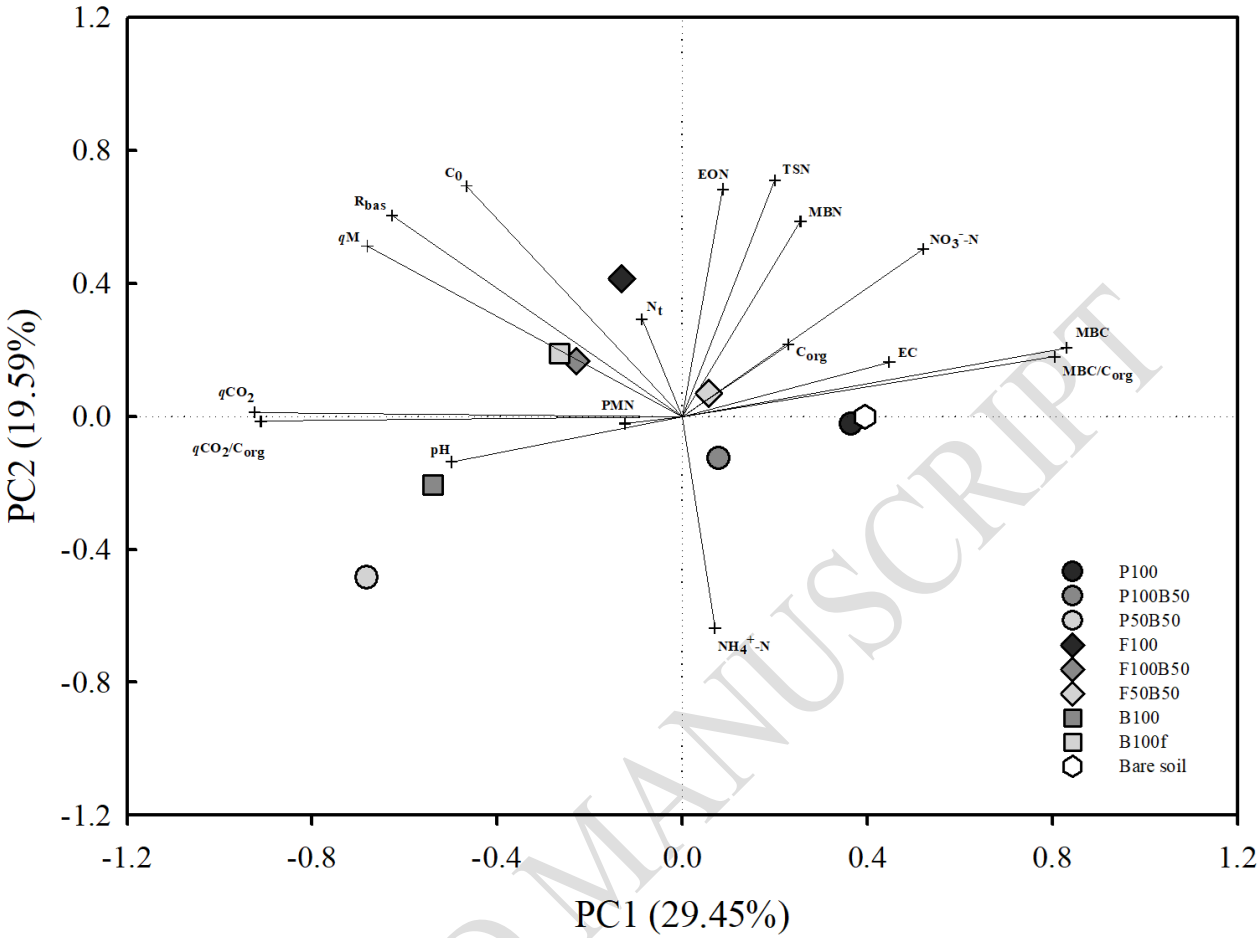

Fig. 5. PCA ordination biplot (PC1 *vs* PC2) of the 17 soil chemical and biochemical variables (loadings) measured in the DW field plots following the nine IC treatments (scores) in a two-year crop rotation. Samples from the four sampling times during the 2011/2012 cropping season were considered all together in the PCA analysis. The biplot has the same origin for scores and loadings.

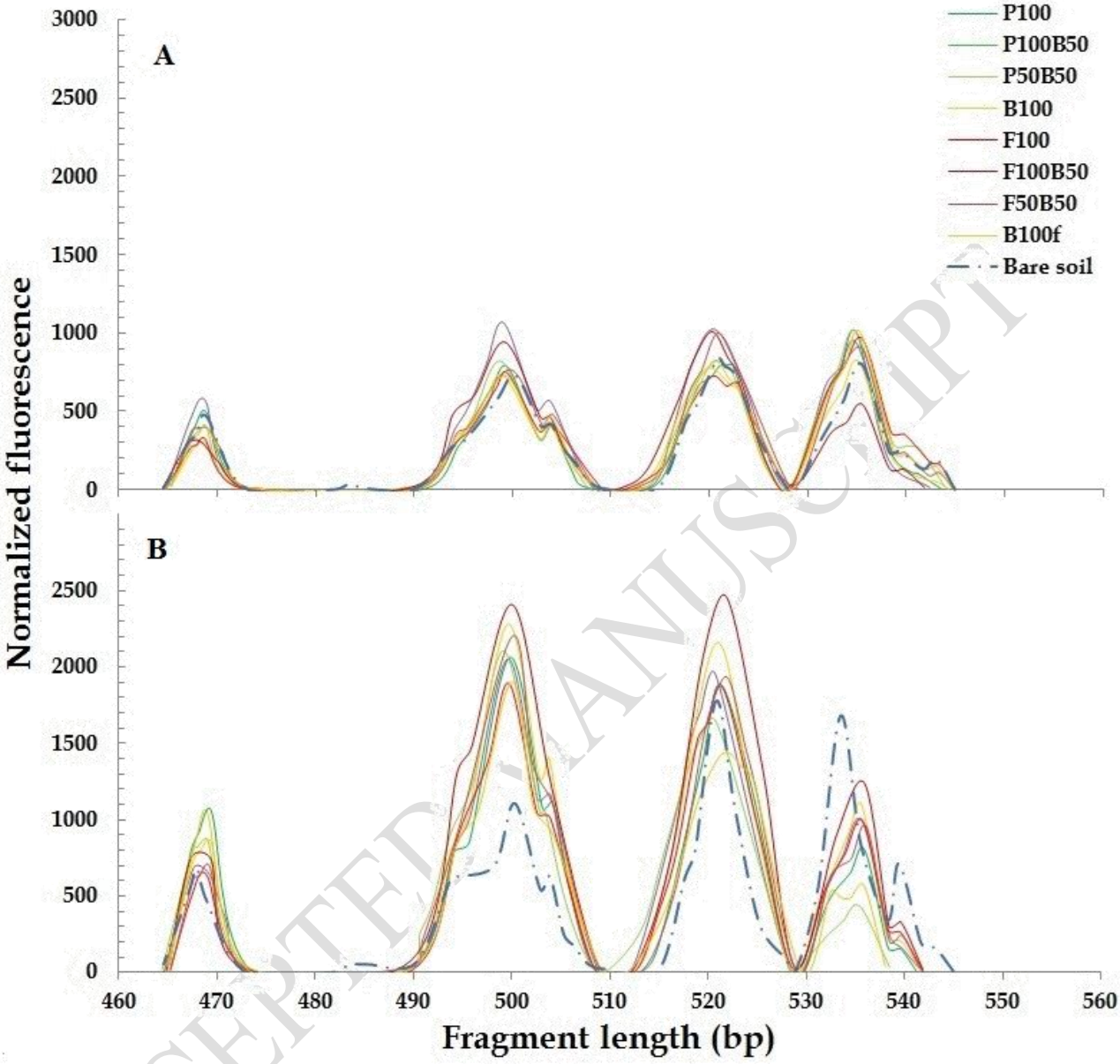

Fig. 6. Curve-based bacterial community profiles in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Here there were considered soil samples taken at the pre-sowing (A) and the harvest (B) stage during the 2011/2012 cropping season. Each profile is the arithmetic average of three-replicated LH-PCR community fingerprints, normalized by total fluorescence intensity.

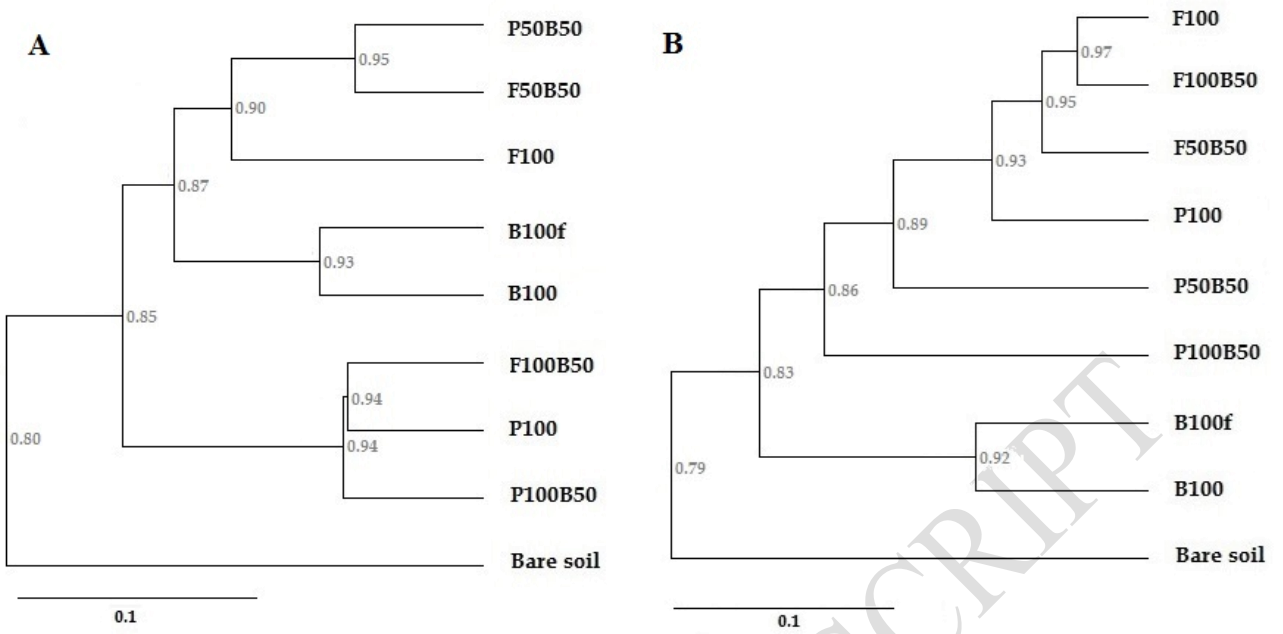

Fig. 7. Dendrogram of hierarchical classification (Dice similarity coefficient, UPGMA clustering method) of molecular banding patterns generated by LH-PCR analysis PCR-amplified 16S rRNA gene-coding fragments from soil-extracted bacterial community DNA monitored in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Here there were considered soil samples taken at the pre-sowing (A) and at the harvest (B) stage during the 2011/2012 cropping season.

Appendix A. Supplementary data

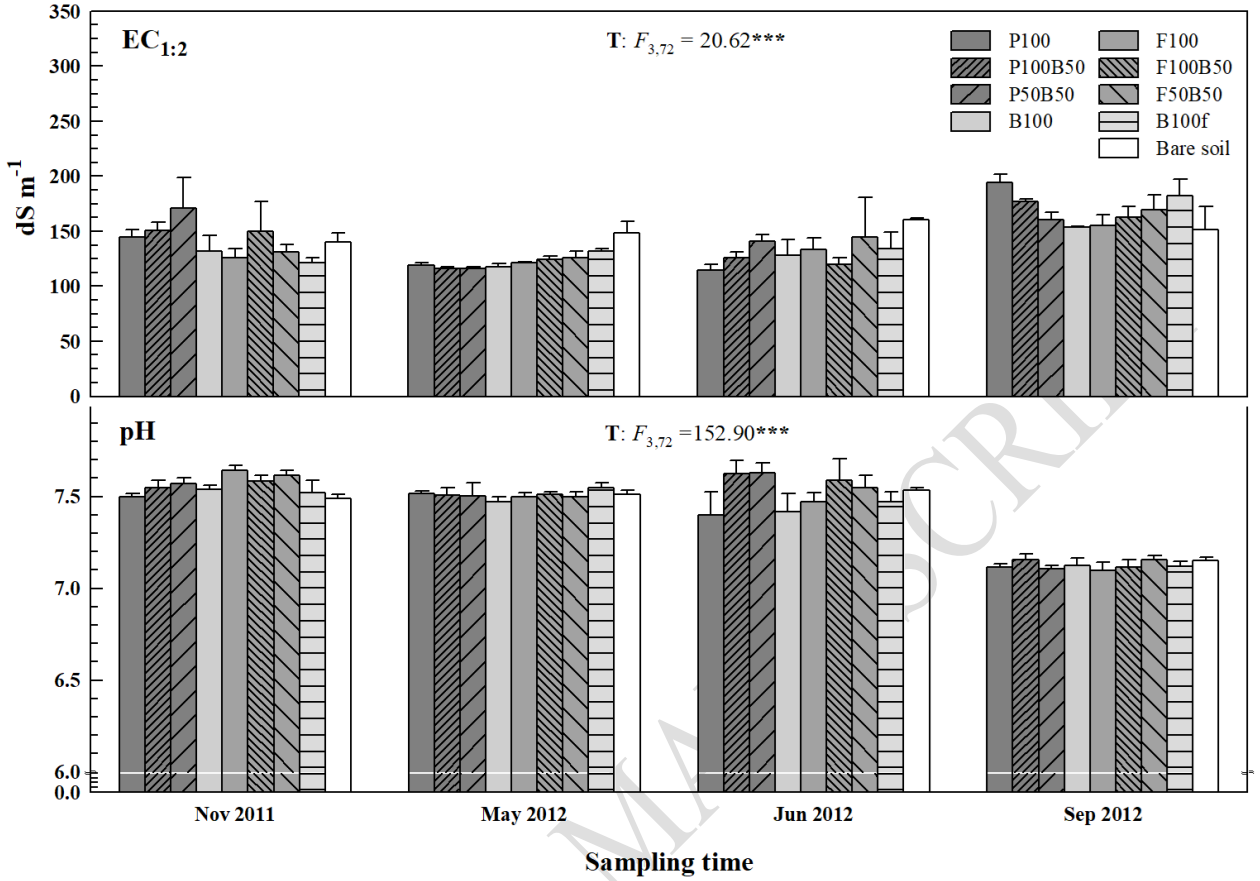

Fig. S1. Changes in the electrical conductivity ($EC_{1:2}$ in 1:2 soil-to-water extracts) and soil pH (mean ± SD, *n=3*) in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at four stages (pre-sowing, ear emergence, harvest and post-harvest) during the 2011/2012 cropping season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $P < 0.05$). Significant effects due to time (T), preceding crop (pC) and their interaction are presented as *F*-values and level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) estimated by a two-way ANOVA (time x preceding crop).

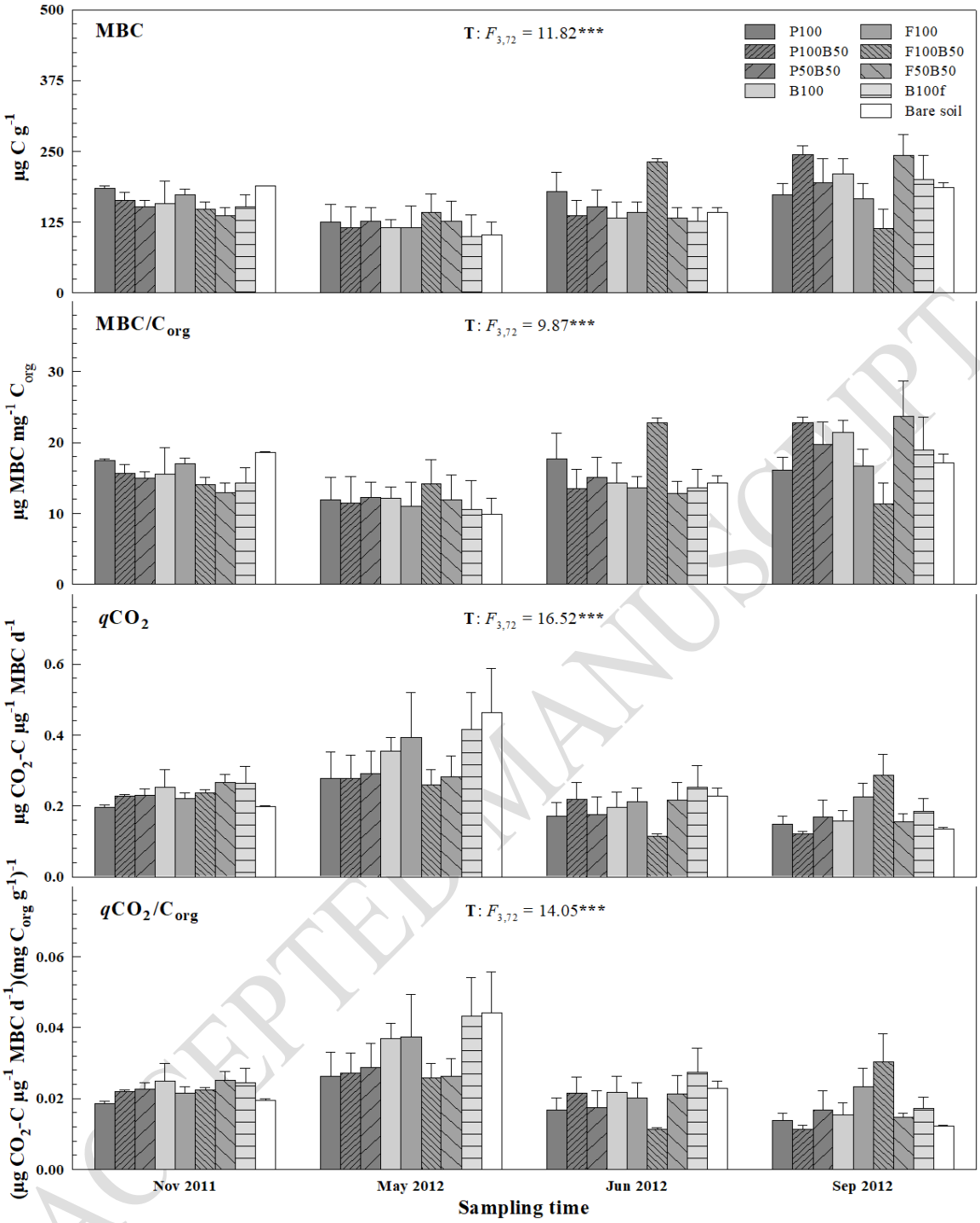

Fig. S2. Changes in soil microbial biomass C (MBC), microbial quotient (MBC/C_{org}), metabolic quotient (qCO_2) and qCO_2/C_{org} ratio (mean ± SD, *n=3*) in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at four stages (pre-sowing, ear emergence, harvest and post-harvest) during the 2011/2012 cropping season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $P < 0.05$). Significant effects due to time (T), preceding crop (pC) and their interaction are presented as *F*-values and level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) estimated by a two-way ANOVA (time x preceding crop).

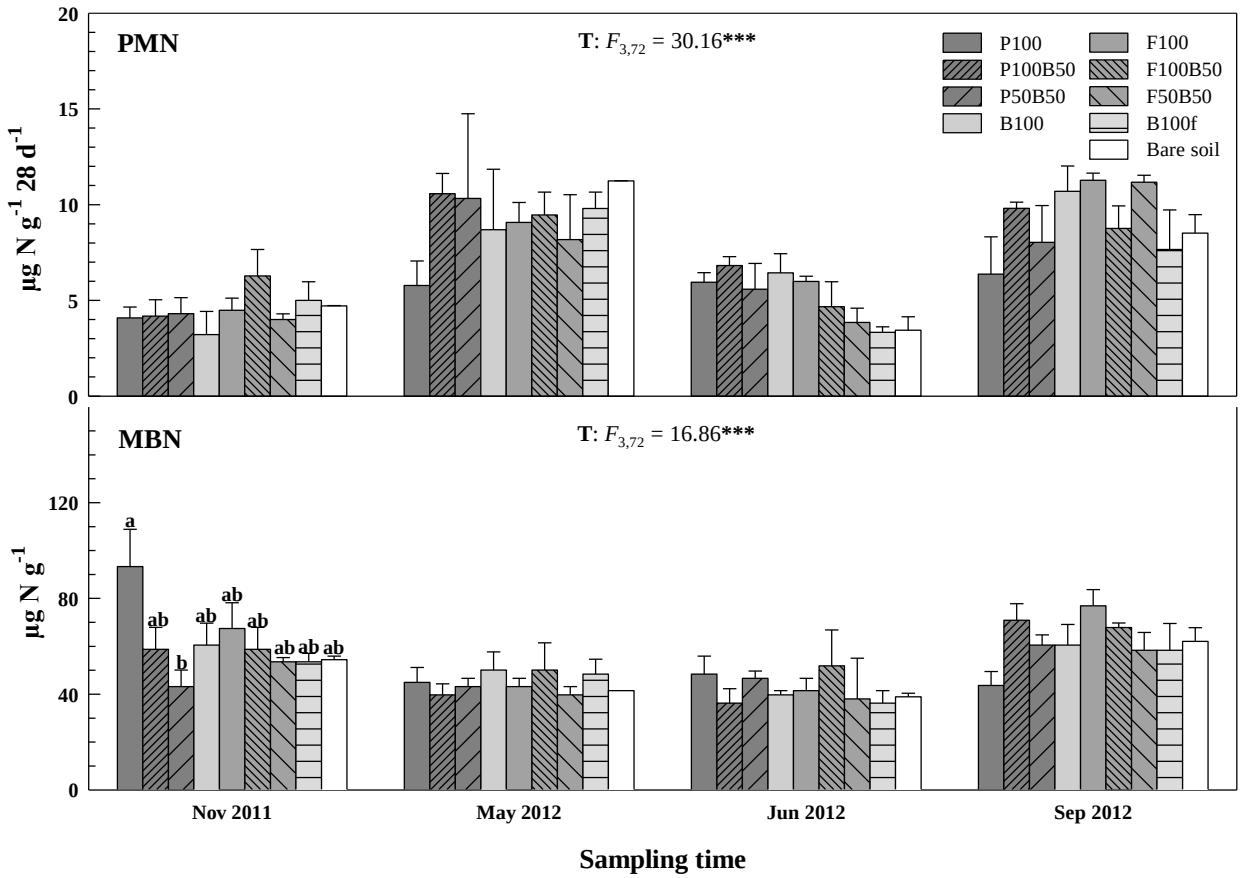

Fig. S3. Changes in soil potentially mineralizable N (PMN) and microbial biomass N (MBN) (mean ± SD, *n=3*) in the DW field plots following the nine IC treatments (P100, P100B50, P50B50, B100, F100, F100B50, F50B50, B100f and Bare soil as in § 2.2) in a two-year crop rotation. Soil samples were taken at four stages (pre-sowing, ear emergence, harvest and post-harvest) during the 2011/2012 cropping season. Within each sampling period, different letters indicate significant differences among treatments (Tukey's HSD test, $P < 0.05$). Significant effects due to time (T), preceding crop (pC) and their interaction are presented as *F*-values and level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) estimated by a two-way ANOVA (time x preceding crop).