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Increasing Legume Input through Interseeding Cover Crops: Soil and Crop Response as Affected by Tillage System

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Abstract: Legumes provide important benefits in rotations. Interseeding cover crops (CCs) allows an additional legume CC in case of a short window after the main crop. However, legume input level and management could modify the expected benefits. In a Mediterranean irrigated agroecosystem, we evaluated the responses of topsoil (0–10 cm) and early maize development to increasing legume CC input in a biannual maize–wheat rotation under traditional tillage (TT; CC incorporated) and minimum tillage (MT; CC rolled-crimped). In the third year, at two early maize stages, we tested three legume input levels: (i) R0, non-CC; (ii) R1, barley–vetch CC; (iii) R2, vetch interseeded into maize in addition to the CC mixture. Overall, MT enhanced soil properties, but frequently conditioned to legume input level. The tillage system affected R1 the most, with MTR1 showing the better overall soil response while TTR0 showed the poorest. MTR2 was the best combination for early maize development, but not for soil health. Moreover, a better overall soil health did not lead to a better early maize performance in the short term. In this alkaline soil, CC favored early maize growth, whereas mycorrhization, enhanced under TT, favored crop nutrition. Increased legume input under MT should be monitored to avoid negative effects in soil in the mid–long term.

Keywords: maize; roller crimper; incorporation; soil health; mycorrhizal fungi; CC mixture

1. Introduction

The sustainable management of agricultural soils is essential to ensure soil ecosystem functions and food security and reduce environmental damage, with rotation diversification, reduced tillage and nitrogen fertilization adjustment being some of the recommended practices [1,2]. The use of cover crops (CCs) in rotation has benefits such as reduction in erosion and N leaching, atmospheric N fixation, increased organic matter (OM) and overall soil health, as well as climate change mitigation and adaptation [3,4]. As legumes contribute to increasing the sustainability of agricultural systems [5], legume CCs are of particular interest. Thus, their ability to fix atmospheric N helps to reduce synthetic N fertilization [6], which is highly appreciated for mitigating environmental effects in irrigated systems. Other advantages are the rapid mineralization of their residues [7] and the stimulation of soil microbial activity, including that of the arbuscular mycorrhizal fungi (AMF) [8].

Legume CCs can enter into rotations in several ways. Typically, they are between two consecutive main crops such as winter CCs. They can also partially overlap with the main crop in 'interseeding', which consists of sowing the CC between the rows of the main crop when the latter is already established. The practice of interseeding allows an earlier establishment of the CC, which is of interest when the period without the main crop is relatively short. The controlled presence of an interseeded CC allows controlling weed development, thereby reducing herbicide use [9] or reducing nitrate leaching [10]. However, a CC interseeded into the main crop may compete with it in reducing yield, either by an inadequate interseeding timing, e.g., in maize [11], or by an incomplete CC termination, e.g., in soybean [10]. When the main crop is a grass crop such as maize, it is common to use a legume CC to fix atmospheric N so that it can be utilized by the main crop [12]. In semi-arid climates, the sowing of legume CCs interseeded into irrigated maize has been successfully tested [13] so that the use of this technique allows intensifying irrigated rotations with a greater input of legumes into the agroecosystem. However, in this type of environment, which combines high temperatures with water availability, there is little information on its impact on soil health or its legacy effects on the main crop nutrition.

The combination of legumes with grasses can be a solution when legume CCs show a lower capacity to build C stocks or improve soil physical properties [14,15], or even when there is no improvement at all [16]. Grass–legume mixtures have been successfully applied for soil conservation [17], also to ensure stable N accumulation and reduce the risk of nitrate leaching [18], or to improve soil organic C accumulation [15], with the added advantage of improving the yield of the main crop [19]. Thus, different grass–legume mixtures have been successfully tested on maize [20]. Combining a grass–legume mixture with an interseeded legume can be a good strategy to increase the input of legumes into the agroecosystems, especially when there is little time to establish a CC between one main crop and another.

Tillage can modulate soil and crop response to the input of CCs into the system [19]. In some cases, tillage may counteract the extent of the ecosystem service benefits provided by CCs [21]. Conversely, CC can mitigate the adverse effects of tillage, e.g., on soil microbiota [22]. Combining CC with conservative tillage is considered beneficial for soil health [23,24] and is a strategy to maintain yields when tillage is reduced [25,26]. Closely related to the type of tillage is the method of CC termination with the corresponding residue management, greatly affecting soil health [22,27]. Thus, CC termination can involve physical or chemical means, and residues can be incorporated or left on the surface as mulch. A traditional method of CC termination is mowing and the subsequent incorporation into the soil by tillage. In conservation agriculture, herbicides are frequently used to terminate CC and, more recently, the roller crimper, which cuts the stems at different heights, forming a layer of residues on the surface [28]. In maize, the termination method of the precedent CC can not only greatly affect crop development and yield [29], but also the soil microbial properties, being this effect on soil stronger under optimal soil moisture conditions [30], which are those of irrigation. Therefore, the way CCs are terminated together with their residue management can modulate the expected benefits of using CCs in rotations.

Few studies have evaluated the combination of different modes of legume CC input into Mediterranean irrigated rotations from the point of view of their integral effect on soil health or the performance of the subsequent main crop. Yet, it is not well known how these effects can be modulated by the tillage system and the CC termination method. Since the legacy effects of CC are most apparent at early stages of the main crop development [31], our objective was to evaluate the response of the soil, as well as that of the subsequent main crop at early growth stages to different levels of legume input with different CC residue management associated with the soil tillage system. For this purpose, we set up a field experiment in an irrigated Mediterranean maize–wheat rotation with three levels of legume CC input and two tillage systems involving contrasting CC termination methods, incorporation and surface disposal. We assessed soil heath through a selection of physical, chemical and biological soil properties, together with growth and nutritional parameters of the subsequent maize. We hypothesized that: (i) a higher legume CC input in the rotation would provide more benefits to the soil and to the main crop and (ii) the combination with reduced tillage would favor soil health as well as crop development.

2. Materials and Methods

2.1. Study Area and Experimental Design

The study was conducted in a field trial at "La Canaleja" experimental farm (40°30′44″ N, 3°18′55″ W, 600 m a.s.l.) in the central Iberian Peninsula. The area has a semiarid Mediterranean climate, Bsk (Köppen–Geiger), with a mean annual temperature of 13.5 °C and a mean annual rainfall of 389 mm (range 186–547 mm), mainly occurring in autumn and spring and almost negligible in summer. The site was in a well-drained river terrace (<2% slope) with a soil classified as Typic Calcixerept (Soil Survey Staff 2014)/Calcic Cambisol (IUSS Working Group WRB 2015). Soil was deep with a sandy loam texture up to ~100 cm in depth with rock fragments < 1%. Selected properties of the topsoil layer (0–20 cm) were: alkaline pH (7.9; 1:2.5 soil:water), low soil organic carbon (8 g C kg^{-1}), low calcium carbonate (74.3 g $CaCO_3$ kg^{-1}), 66% sand (0.05–2 mm), 20% silt (0.002–0.05 mm) and 14% clay (<0.002 mm).

The experiment was established in 2018 on a bi-annual irrigated maize–wheat rotation with two study factors and four field replications. In factor 1, two modes of introducing legume CC, one as winter CC and one with an additional interseeded CC, were evaluated and compared with a non-CC mode (no legume; R0). The winter mode consisted of a mixture of barley and vetch included in a temporary succession between the spring wheat (2019) and the third-year maize (2020) (one legume CC; R1). In the third mode, a vetch CC interseeded into the rows of the first maize (2018) was included in addition to the winter mixture CC, so that in two agricultural years, two main crops and two CC were introduced in the rotation (two legume CCs; R2) (Figure 1). Factor 2 was the soil tillage system with two types, minimum tillage (MT) and traditional tillage (TT), which were implemented 6 years before the start of the present experiment. The CC termination was different depending on the tillage system. In TT, the CCs were terminated with a brush cutter and buried with a moldboard to 30 cm depth. In ML, the interseeded CC was chopped and lightly buried with a chisel and the winter mixture CC was terminated with a roller crimper, leaving the residues on the surface. The six crossed treatments (three rotation modes × two tillage systems) were distributed in 24 plots of 8 × 16 m^2.

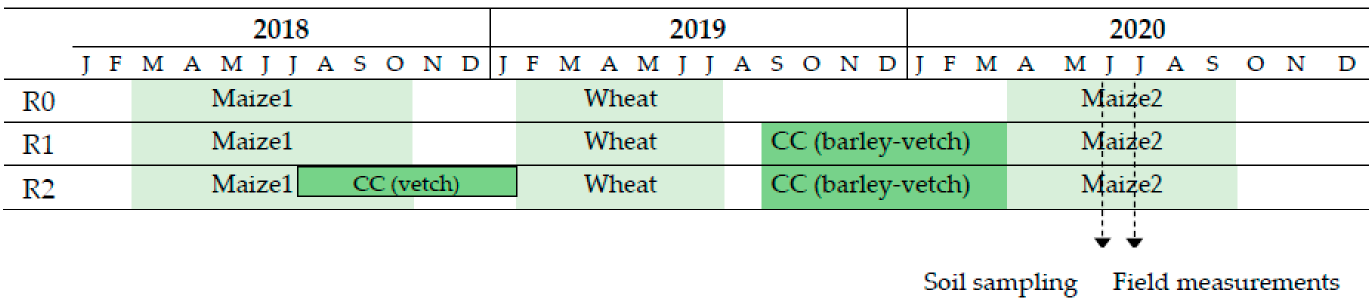

Figure 1. Scheme of rotations. R0: maize–wheat rotation with no cover crop (CC); R1: rotation with winter barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 before wheat plus barley–vetch CC between wheat and maize2.

2.2. Experiment Management

Before the experiment establishment (April 2018), the area was homogenized with canola (*Brassica napus* L.). Before maize sowing, P and K fertilizations were applied (70 kg ha^{-1} P_2O_5 and 120 kg ha^{-1} K_2O, as triple superphosphate, 46%, and potassium chloride, 60%, respectively) to the soil. In the TT plots, a moldboard (30 cm depth) followed by a disc harrow (10 cm depth) was previously passed over to prepare the soil for sowing, whereas in MT, a chisel was used only to 10 cm in depth. Maize (*Zea mays* L., Pioneer P1574 variety) was sown on 14 May 2018 (80,000 plants ha^{-1}) at a row spacing of 75 cm.

A dose of 87.5 kg N ha^{-1} was applied twice (June and July) based on a previous soil analysis. Irrigation was applied by a sprinkler delivery system (16 m × 16 m) with a water dose based on the crop evapotranspiration (using the FAO Penman–Monteith as reference evapotranspiration) and a washing fraction to avoid soil salinity.

The vetch CC (*Vicia sativa* L., Aitana variety) interseeded into maize was sown at a rate of two million seeds per ha (70 kg ha^{-1}) on the same date as the second N fertilizer was applied. A cultivator (5 cm depth) was passed to bury the fertilizer, vetch seeds and emerged weeds. The vetch CC was cultivated until end of January 2019 when it was terminated by chopping and left on the ground. In the TT plots, vetch residues and small weeds were buried into 30 cm depth with a moldboard. In the MT plots, a chisel was used to 10 cm depth, leaving most vetch residues above the surface.

The spring wheat (*Triticum aestivum* L., Badiel variety) was sown in February 2019 at a rate of 230 kg ha^{-1} (around 5 million seeds per ha). It was fertilized with 75 kg ha^{-1} N in early May. The wheat was watered to make up the difference between potential evapotranspiration and actual precipitation. In early October 2019, a CC mixture was sown (100 kg ha^{-1}) with 50 kg ha^{-1} vetch (*Vicia sativa* L., Aitana variety) and 50 kg ha^{-1} barley (*Hordeum vulgare* L., Vinagrosa variety). CC did not receive any fertilizer or irrigation during its growing period. In mid-October of 2019, 2% glyphosate was applied to the non-CC plots. The CC mixture was terminated at the end of March 2020 using different methods. In the TT plots, the CC mixture and weeds were chopped, and the residue was buried into 30 cm soil depth with a moldboard; in the MT plots, the CC mixture and weeds were terminated with a roller crimper, leaving the residues covering the soil surface. In both systems, small weeds in R0 plots were chopped and managed according to the corresponding tillage system.

In May 2020, the maize of the second rotation cycle was sown (80,000 plants ha^{-1}) directly through the CC residues in the MT plots and after a harrow pass in the TT ones. In early June 2020, a first N fertilization (85 kg ha^{-1}) was applied. In mid-2020, nicosulfuron was applied as a post-emergence maize herbicide for weed control. Interseeded vetch was sown (70 kg ha^{-1}) and a second N fertilization (87.5 kg ha^{-1}) was applied by end of June 2020, both buried with a cultivator to a 5 cm depth, together with emerged seeds.

2.3. Soil and Plant Sampling and Analyses

Once the first two-year cycle was completed, sampling and field measurements were carried out at the beginning of the second maize (2 June 2020) 25 days after sowing (DAS) maize, not long before the first N fertilization. Three maize plants with their root systems and soil were randomly collected from each plot (cores of 8 cm diameter and 10 cm depth). At the sampling time, plants showed 3–4 leaves completely unfolded. Samples were kept in refrigerated conditions and transported to a laboratory in their own farm. The three samples per plot were pooled to obtain a composite soil and plant sample from each plot. Plants were carefully separated from the soil and then roots and shoots were separated and washed with tap water and then distilled water. After weighting the fresh root biomass, fine roots were selected and stored in a 50% ethanol solution at 4 °C for AMF colonization measurement.

The soil samples were homogenized and divided into two parts: one part was frozen at −20 °C for biological analyses and the other was air dried, sieved (<2 mm) and stored at room temperature for physicochemical measurements. When necessary, soil samples were milled in a ball mill. Soil pH and electrical conductivity at 25 °C ($EC_{1:2.5}$) were measured in 1:2.5 (*w*/*v*) of soil/water suspension; total organic carbon (TOC) was measured by wet oxidation method [32]; soil N concentration was determined using the Kjeldahl method [33]; particulate (POC) and mineral-associated organic carbon (MAOC) were measured following Cambardella and Elliot [34]. For mineral N (Nmin), 2M KCl soil extracts (5 g of soil: 50 mL of KCl) were analyzed for $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ (FIAstar™ 5000, FOSS Analytical AB, Höganäs, Sweden) following the methods of Griess–Ilosvay and Solorzano [35,36], respectively. Extractable soil elements were analyzed by ICP-OES (iCAP 6500-duo spectrometry, Thermo Elemental Co. Iris Intrepid II XDL, Waltham, MA, USA) in extracts obtained by shaking 3 g of air-dried soil with 30 mL of Mehlich III solution. Water-stable aggregates (WSA) were measured by wet-sieving of air-dried 1–2 mm aggregates through a 250 mm sieve [37].

Before microbial analysis, frozen samples were incubated for 7 days at 22 °C and 60% of water holding capacity. Soil basal respiration (BR) was measured by CO_2 quantification in an alkaline trap after incubation for 24 h at 22 °C [38]. Substrate-induced soil respiration (SIR) was obtained by the same procedure after adding glucose (3:1 ratio talc to glucose) and incubation for 4 h at 22 °C [38,39]. Microbial biomass C and N (MBC, MBN) were obtained by the fumigation–extraction method [40]; carbon concentration in the extracts was determined by back titration redox based on Yakovchenko y Sikora [41] and N was measured with a total nitrogen analyzer (TOC-V CSH/TNM-1, Shimadzu Scientific Instruments, Kyoto, Japan). The length of the extraradical hyphae (mycelium) was determined from a soil suspension (2 g soil) sequentially sieved through 250 and 50 µm sieves based on the membrane filter technique and the gridline intersect method as described by García-González et al. [42].

Maize shoots were dried at 60 °C for 72 h, weighted and ground in a ball mill for subsequent nutrient analysis. The shoot N concentration was determined using the Kjeldahl method [33] and other elements were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES; iCAP 6500-duo spectrometry, Thermo Elemental Co. Iris Intrepid II XDL). Roots were cleared with 10% KOH and then stained with ink-vinegar solution [43]. The magnified intersections method was used to obtain the percentage of AMF colonization [44].

At 55 DAS maize (2 July 2020), 10 plants were selected at random from each plot to measure maize height and chlorophyll concentration using a SPAD-502® (Konica Minolta Inc., Tokyo, Japan). In addition, three soil cores (5 cm Ø, 5 cm height) were randomly sampled from each plot at 0–5 cm depth. Bulk density of the fine earth (BD) was obtained after correcting for rock fragments larger than 2 mm [45].

2.4. Data Analysis

An analysis of variance was applied with a linear model for a factorial randomized design. The rotation type with different levels of legume CC inputs, the tillage system and their interaction were considered fixed effects. One plot was removed from the analysis due to a very high, nonrepresentative calcium carbonate content. The normality and homoscedasticity of the data were verified. A Box–Cox transformation was applied to the variables that required transformations, so that a logarithm transformation was used for the next soil properties: POC, POC/TOC, C/N, RB, SIR, Nmin, Fe, Mn, Zn and crop nutrients Fe and Cr. Differences between means were evaluated with LSD's test for a p-value < 0.05. We obtained the Pearson's product–moment and their significance levels to assess bivariate relationships between the soil properties and the crop variables. A redundancy analysis (RDA) was performed to relate different subgroups of soil properties (physicochemical, microbial and nutritional) to treatments, which were the combinations of the three levels of the rotation factor and the two levels of the tillage system. Given the soil spatial heterogeneity we found, RDA was also performed to relate crop variables to treatments in a first step, to soil properties in a second step, and finally to both groups together. Both response and explanatory variables were transformed to ensure linearity and symmetry and standardized. Given the high number of soil variables and the multicollinearity between them, the variance inflation factor was obtained firstly to remove variables highly correlated, and secondly, a Monte Carlo permutation test was conducted using 999 permutations for a forward, backward and stepwise forward procedure to select soil variables (p-value < 0.05). The soil properties resulting from the selection procedure were used in the final RDA together with the treatments to explain the crop variables. A variance partitioning analysis was carried out following a Monte Carlo permutation test to explore how much of the crop response was explained by the treatments and how much by the soil properties outside of the treatments. In a following analysis, conditional effects were tested using treatments as a covariate. The significance level was set at $p \leq 0.05$. Analyses were performed with the software R version 4.2. [46]. The RDA analysis was conducted with the function rda of the vegan package [47] and the RDA plot with the ggplot2 package [48].

3. Results

3.1. Soil and Crop Variables as Affected by Legume Input Level, Tillage System and Its Interaction

The tillage system alone influenced soil properties to a greater extent than the level of legume CC input in the rotation, being relevant the interaction between the two factors, mainly for microbial variables (Table 1). Of the soil variables, only WSA, S and Na (72.4 mg $kg^{-1} \pm 23.0$) did not respond to the factors. Relative to TT, MT increased $EC_{1:2.5}$, TOC, POC, MAOC, POC/TOC, bulk density (BD), mycelium, MBC, MBN, K, Mg, P ($p < 0.1$) and Fe ($p < 0.1$), while decreased pH and Ca ($p < 0.1$), regardless of the rotation type (Table 1). Minimum tillage also increased C/N, basal respiration (BR), substrate-induced respiration (SIR) and Zn conditioned by the level of legume CC input (Table 1; Figure 2). Basal respiration and C/N were higher in MT only at R0, and SIR followed a similar pattern. By contrast, Nmin dropped in MTR0 and peaked at MTR1, while TT plots were not affected by the rotation type. AMF colonization was increased by TT under R0 and R1 but dropped in R2, following an opposite pattern under MT (Figure 2).

Several variables showed a maximum of amplitude for R1 with a peak in MTR1, and frequently, the minimum in TTR1 (Figure 2 and Figure S1). Thus, soil N, Cu and Mn ($p < 0.1$) only showed differences according to the tillage system for R1, so that its concentrations in R1 were enhanced under MT and reduced under TT. In the same line were the patterns of POC ($p < 0.05$) and MBN ($p < 0.1$), with the highest value under MT and the lowest under TT for R1 (not significant for POC/TOC, MAOC). Zinc and, to a lesser extent, MBC (not significant), also peaked at R1MT with a maximum amplitude of values for R1 between MT and TT.

Table 1. Soil physicochemical, microbial and nutritional properties as affected by the level of legume cover crop (CC) input and tillage system at early maize growth stages.

Soil Property	Unit	Rotation (F1)			Tillage (F2)		Factors		
		R0	R1	R2	TT	MT	F1	F2	F1 × F2
			Physicochemicals						
pH 1:2.5		8.24(0.03) a	8.21(0.02) a	8.26(0.03) a	8.30(0.01) b	8.17(0.01) a	ns	***	ns
EC 1:2.5	µS cm^{-1}	297(5.91) a	331(11.9) b	310(8.89) ab	298(5.04) a	327(8.55) b	**	**	ns
TOC	g kg^{-1}	7.83(0.41) a	8.14(0.68) a	7.54(0.50) a	6.61(0.17) a	9.06(0.25) b	ns	***	ns
POC	g kg^{-1}	2.00(0.21) a	2.11(0.32) a	2.05(0.19) a	1.48(0.04) a	2.62(0.11) b	ns	***	*
MAOC	g kg^{-1}	5.31(0.15) a	5.28(0.38) a	5.14(0.18) a	4.71(0.11) a	5.74(0.14) b	ns	***	ns
POC/TOC	%	26.9(1.57) a	27.6(1.93) a	28.1(1.23) a	23.8(0.44) a	31.2(0.67) b	ns	***	ns
C/N		15.0(3.09) a	13.0(1.86) a	15.2(2.85) a	11.5(1.20) a	17.3(2.46) b	ns	*	+
BD	g cm^{-3}	1.44(0.03) b	1.36(0.04) ab	1.34(0.03) a	1.33(0.02) a	1.42(0.03) b	+	*	ns
WSA	%	41.8(1.84) a	44.6(3.65) a	40.8(2.11) a	40.8(1.77) a	44.0(2.25) a	ns	ns	ns
			Microbial						
Mycelium L	cm g^{-1}	20.8(1.54) a	23.7(3.15) a	18.8(1.95) a	17.6(1.21) a	24.6(1.80) b	ns	**	ns
AMF Col	%	32.3(2.17) a	31.7(4.61) a	30.8(3.25) a	34.0(2.59) b	29.2(2.67) a	ns	+	***
BR	mg C-CO_2 $kg^{-1}h^{-1}$	1.22(0.14) a	1.32(0.08) a	1.24(0.04) a	1.13(0.07) a	1.38(0.07) b	ns	**	***
SIR	mg C-CO_2 $kg^{-1}h^{-1}$	5.33(0.60) a	6.24(0.21) b	6.24(0.21) b	5.19(0.34) a	6.69(0.14) b	***	***	***
MBC	mg kg^{-1}	149(7.64) a	178(17.5) b	189(12.23) b	148(7.71) a	197(9.68) b	*	***	ns
MBN	mg kg^{-1}	26.0(1.78) a	28.6(3.59) a	27.3(2.03) a	22.2(1.08) a	32.4(1.52) b	ns	***	+
			Macronutrients						
N	g kg^{-1}	0.62(0.07) a	0.70(0.11) a	0.58(0.07) a	0.62(0.04) a	0.65(0.08) a	ns	ns	*
Nmin	mg kg^{-1}	12.6(1.19) a	17.1(1.61) b	14.1(0.74) b	15.2(0.66) b	14(1.37) a	**	**	***
P	mg kg^{-1}	32.1(1.82) a	28.3(2.55) a	30.1(2.30) a	27.7(1.60) a	32.6(1.73) b	ns	+	ns
K	mg kg^{-1}	227(12.16) a	246(23.1) a	219(8.27) a	208(6.66) a	253(13.1) b	ns	**	ns
Ca	g kg^{-1}	2.34(0.10) a	2.39(0.22) a	2.29(0.09) a	2.51(0.14) b	2.17(0.05) a	ns	+	ns
Mg	mg kg^{-1}	682(29.0) a	648(30.7) a	666(16.1) a	613(13.5) a	717(13.6) b	ns	***	ns
S	mg kg^{-1}	44.4(1.92) a	45.7(1.35) a	41.5(1.64) a	42.9(1.56) a	44.8(1.27) a	ns	ns	ns

Table 1. *Cont.*

Soil Property	Unit	Rotation (F1)			Tillage (F2)		Factors		
		R0	R1	R2	TT	MT	F1	F2	F1 × F2
		Micronutrients							
Cu	mg kg^{-1}	1.92(0.04) a	1.99(0.14) a	1.89(0.05) a	1.90(0.06) a	1.96(0.07) a	ns	ns	*
Fe	mg kg^{-1}	42.2(1.13) a	42.5(2.74) a	42.4(1.30) a	40.2(1.66) a	44.5(0.77) b	ns	+	ns
Mn	mg kg^{-1}	163(4.96) a	162(13.0) a	165(4.24) a	156(7.51) a	171(4.09) a	ns	ns	+
Zn	mg kg^{-1}	1.45(0.10) ab	1.53(0.23) b	1.21(0.07) a	1.13(0.05) a	1.65(0.12) b	+	***	*

R0: Rotation with non-CC; R1: rotation with barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 plus barley–vetch CC between wheat and maize2; TT: traditional tillage; MT: minimum tillage; EC: electric conductivity; TOC: total organic carbon; POC: particulate organic carbon; MAOC: mineral-associated organic carbon; C/N: carbon/nitrogen ratio; BD: bulk density; WSA: water-stable aggregates; AMF Col: arbuscular mycorrhizal fungi colonization; BR: basal respiration; SIR: substrate-induced respiration; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen; Nmin: mineral nitrogen; all soil nutrients are Mehlich III-extractable except N and Nmin; in parenthesis, the standard error of each mean; different letters indicate significant differences between means according to LSD test (*** p-value < 0.001; ** < 0.01; * < 0.05; + < 0.1; ns: not significant).

On the other hand, $EC_{1:2.5}$, MBC and BD ($p < 0.1$) responded to the legume CC input level irrespective of the tillage system: CC increased $EC_{1:2.5}$ and MBC, with no differences between R1 and R2, while R2 reduced BD compared to R0 (Table 1).

In general, the tillage system and the legume CC input level independently influenced the early maize development variables (25 and 55 DAS) (Table 2). Maize growth at 25 DAS was higher after CC than after bare fallow, with the highest height in R2 under both MT and TT. At 55 DAS, the differences in height were not significant, but chlorophyll value increased in R2 compared to R0. Minimum tillage increased height and root biomass at 25 DAS compared to TT. Overall, early maize development variables showed better results, combining reduced tillage and CC use.

Table 2. Maize growth variables and element concentrations in maize shoots as affected by legume CC input level and tillage system at early growth stages.

Crop Variables	Unit	Rotation (F1)			Tillage (F2)		F1	F2	F1 × F2
		R0	R1	R2	TT	MT			
		Development variables							
Height 25DAS	cm	37.4(1.52) a	40.9(1.01) b	44.1(1.12) a	38.9(1.16) a	42.7(1.18) b	**	**	ns
Shoot biomass 25DAS	g plant^{-1}	3.32(0.32) a	4.30(0.19) b	4.51(0.20) b	4.00(0.31) a	4.09(0.19) a	**	ns	*
Root biomass 25DAS	g plant^{-1}	5.45(0.38) a	6.78(0.31) b	6.62(0.27) b	5.88a(0.20)	6.68(0.37) b	**	*	ns
Height 55DAS	cm	83.9(2.56) a	87.0(5.24) a	90.2(3.95) a	89.5(2.69) a	84.6(3.47) a	ns	ns	ns
Chlorophyl 55DAS	%	42.4(0.90) a	44.5(0.86) ab	46.9(0.86) b	45.2(0.68) a	44.0(1.02) a	**	ns	ns
		Macronutrients							
N	g 100 g^{-1}	4.20(0.17) a	4.06(0.13) a	4.35(0.10) a	4.39(0.11) b	4.01(0.09) a	ns	**	ns
P		0.37(0.02) a	0.35(0.01) a	0.37(0.01) a	0.36(0.01) a	0.37(0.37) a	ns	ns	ns
K		4.15(0.19) a	4.10(0.14) a	3.96(0.12) a	4.27(0.12) b	3.87(0.09) a	ns	*	ns
Ca		0.50(0.004) b	0.46(0.010) a	0.49(0.007) b	0.48(0.005) a	0.49(0.008) a	*	ns	ns
Mg		0.56(0.02) a	0.55(0.02) a	0.61(0.02) a	0.53(0.01) a	0.61(0.02) b	ns	**	ns
S		0.28(0.011) a	0.28(0.005) a	0.29(0.006) a	0.28(0.005) a	0.28(0.007) a	ns	ns	ns
		Micronutrients							
B	mg kg^{--1}	7.34(0.25) a	7.02(0.27) a	7.05(0.16) a	7.60(0.12) b	6.67(0.13) a	ns	***	ns
Cu		8.82(0.23) a	9.01(0.16) a	9.18(0.26) a	9.19(0.23) a	8.81(0.10) a	ns	ns	ns
Fe		757(144) a	622(121) a	526(50.8) a	706(67.4) b	564(109) a	ns	+	+
Mn		91.3(3.80) b	81.7(5.01) a	82.5(3.23) a	94.6(1.71) b	76.0(2.22) a	**	***	ns
Mo		0.42(0.02) b	0.29(0.03) a	0.27(0.01) a	0.34(0.01) a	0.32(0.03) a	**	ns	ns
Zn		38.1(2.24) a	45.8(2.66) a	38.9(2.37) a	41.8(2.59) a	40.1(1.67) a	ns	ns	ns
		Potentially toxic elements							
Pb	mg kg^{-1}	2.97(0.28) a	2.55(0.24) a	2.41(0.12) a	2.79(0.12) a	2.49(0.23) a	ns	ns	ns
Cd		0.07(0.003) a	0.06(0.004) a	0.07(0.000) a	0.06(0.001) a	0.07(0.002) b	ns	**	*
Cr		2.04(0.35) a	1.79(0.25) a	1.56(0.09) a	1.78(0.16) a	1.82(0.24) a	ns	ns	*

R0: Rotation with non-CC; R1: rotation with barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 plus barley–vetch CC between wheat and maize2; TT: traditional tillage, MT: minimum tillage; DAS: days after sowing; in parenthesis, the standard error of each mean; different letters indicate significant differences between means according to LSD test (*** p-value < 0.001; ** < 0.01; * < 0.05; + < 0.1; ns: not significant).

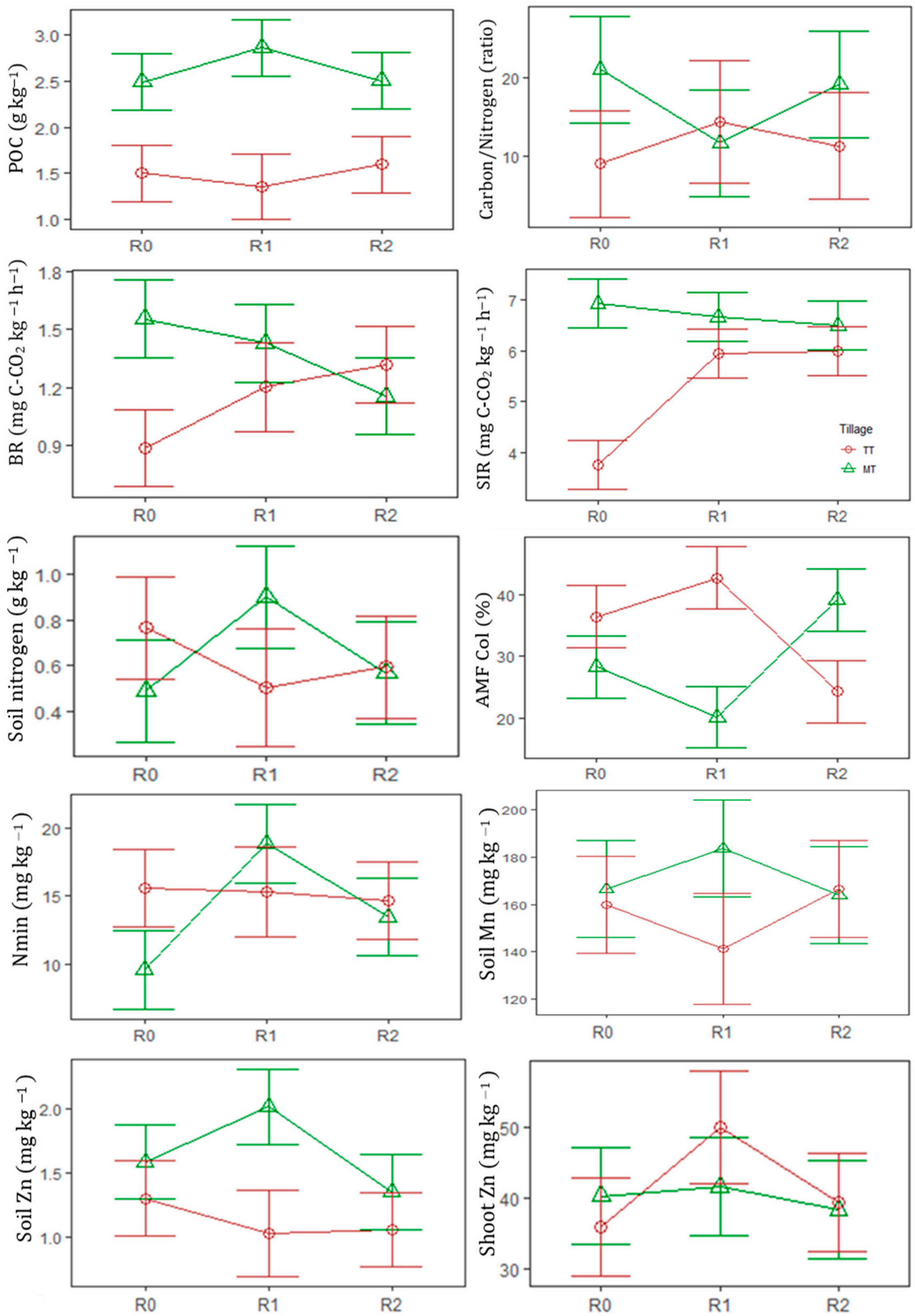

Figure 2. Particulate organic carbon (POC), carbon/nitrogen ratio, basal respiration (BR), substrate-induced respiration (SIR), soil nitrogen, arbuscular mycorrhizal fungi colonization (AMF Col), mineral nitrogen (Nmin), soil Mn, soil Zn and shoot Zn, as affected by legume CC input level and tillage system interaction; R0: rotation with non-CC; R1: rotation with barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 plus barley–vetch CC between wheat and maize2; TT: traditional tillage; MT: minimum tillage.

Traditional tillage increased the concentrations of N, K, B, Mn and Fe ($p < 0.1$) in maize shoots, while MT increased shoot Mg as well as Cd (Figure S1). Non-CC (R0) increased shoot Mn and Mo, R1 decreased shoot Ca and R2 tended to increase shoot Mg. The tillage system only had an effect on R1 for shoot Fe, which peaked at TTR1 (Figure S1), concurrent with high AMF colonization values, in line with patterns shown by shoot Zn (Figure 2), Cr and Pb.

For the whole dataset, OM variables were positively correlated to $EC_{1:2.5}$ (r = 0.63 ** with TOC and POC) and $EC_{1:2.5}$ with Nmin (r = 0.55 **). MBC correlated positively with OM variables, $EC_{1:2.5}$, soil nutrients such as K, Mg and Zn, as well as with early maize growth variables and shoot Cd. BR was negatively correlated with shoot N (r = −0.55 **) and positively with soil Mg (r = 0.53 **). AMF colonization correlated negatively with POC/TOC (r = −0.47 *). Under TT, this negative correlation of AMF colonization with POC/TOC increased (r = −0.77 **), while correlated positively with shoot Fe (r = 0.67 *); BR negatively correlated with TOC (r = −0.47 *) and soil Zn (r = −0.61 *) and positively with shoot biomass (r = 0.80 **). Under MT, MBC positively correlated with Nmin (r = 0.79 **) and BR negatively correlated with height and shoot biomass (r = −0.75 ** and r = −0.64 **, respectively).

3.2. Soil Response to Treatments by Type of Variables—A Comprehensive Soil Response

Treatments explained 56% of the variability of soil physicochemical variables (Figure 3a). The tillage system led to the greatest differences in these variables. Within MT, R1 differed from R0 with R2 in an intermediate position, whereas within TT, there was no difference between the three rotations. As indicated previously, higher values of OM variables, $EC_{1:2.5}$ and BD were related to MT, whereas TT increased pH in this alkaline soil. With respect to microbial variables, treatments explained 59% of its variation, being soil microbial response to treatments well differentiated with no ellipses overlapping (Figure 3b). In general, the soil microbial variables were enhanced under MT, except for AMF colonization. The low colonization values in TTR2 brought its microbial response closer to that of MTR1 and MTR0. By contrast, MTR2 and TTR1, with high AMF colonization, tended to bring their microbial responses closer together. If AMF colonization is omitted (Figure S2a), the responses of R2 under both TT and MT tended to match and were quite similar to that of R1TT. The rotation showing the lowest values of the microbial variables was the one without CC under TT (TTR0). In contrast to physicochemical and microbial variables, treatments explained only around 25% of the variation in both soil macro- and micronutrients. For soil macronutrients (Figure 3c), differences between the six treatments were relatively marked. K and Mg were higher under MT and Ca increased in TT treatments, with Nmin and N separating by rotation type. The treatments differed less with respect to the micronutrient concentration, with MTR1 having the highest concentrations in soil (Figure S2b).

Overall, considering 10 physicochemical and nutritional variables (Figure S2c), MT resulted in better soil properties than TT. The three rotations resulted in similar soil behavior under TT, whereas under MT, the soil responses were different, with R1 standing out as the best rotation and R2 in an intermediate position. When microbial variables were added to obtain a comprehensive soil response as a soil health proxy (Figure 3d), the pattern under MT remained as above, but under TT, the three rotations differed with TTR0 showing the worst overall soil health.

3.3. Crop Response to Treatments by Type of Variables—A Comprehensive Crop Response

Treatments alone explained ~37% of the variation of the five variables related to early maize development (heights, shoot biomass, root biomass and chlorophyll) (Figure 4a). Maize plants after non-CC showed a lesser overall development. In particular, TTR0 stood out for low development at 25 DAS while MTR0 stood out for low chlorophyll value at 55 DAS. Under TT, the higher the legume input, the more maize development was observed, while under MT, the two treatments with CC showed similar development (Figure 4a). Outside treatments, none of the soil properties were sufficiently relevant to explain the variation in maize development variables.

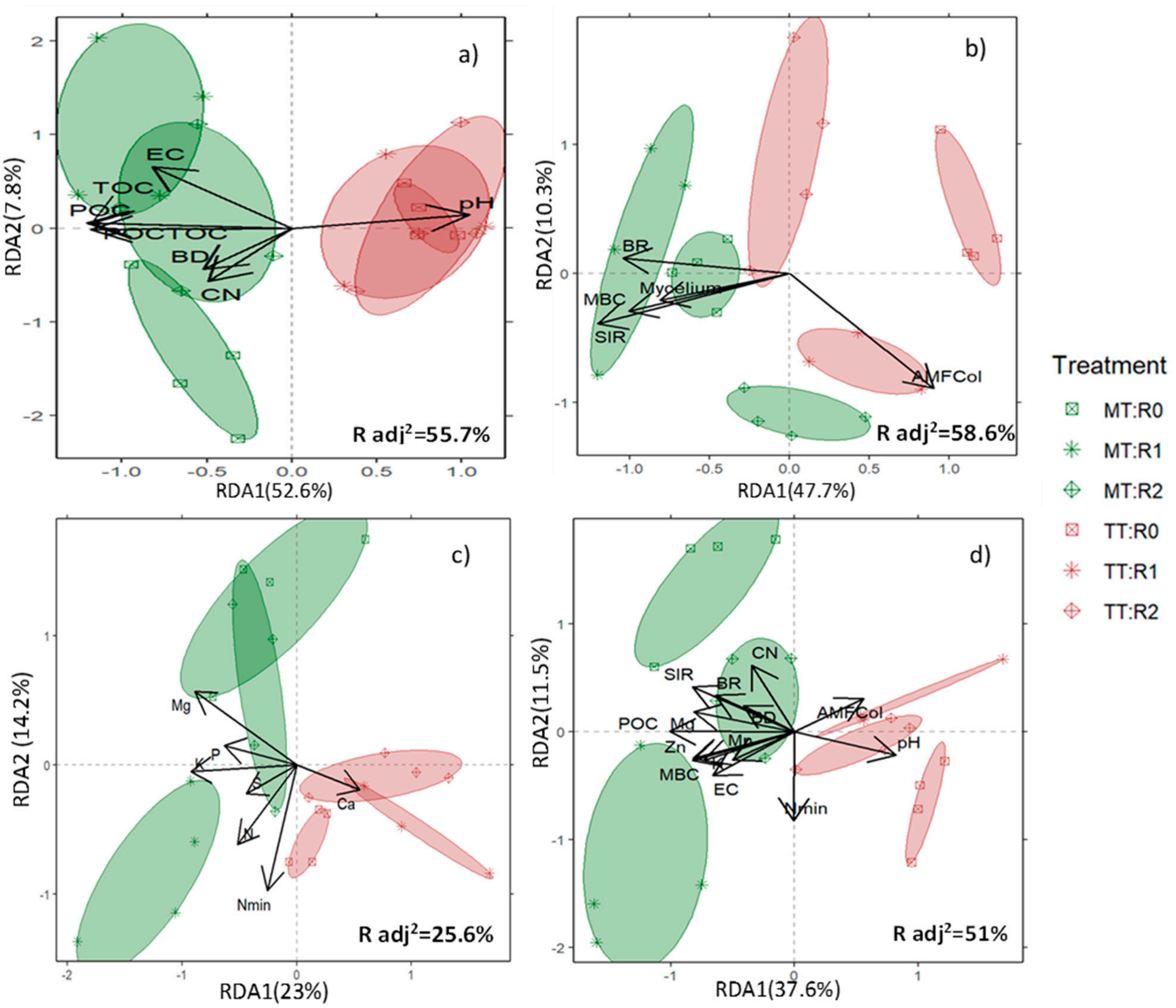

Figure 3. Redundancy analysis (RDA) results to explain: (**a**) soil physicochemical variables, (**b**) soil microbial variables, (**c**) soil macronutrients, (**d**) comprehensive soil response based on 14 variables, from six combined treatments of tillage system (TT: traditional tillage, MT: minimum tillage) and rotation type (R0: rotation with non-CC; R1: rotation with barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 plus barley–vetch CC between wheat and maize2); EC: electric conductivity, BD: bulk density, TOC: total organic carbon, POC: particulate organic carbon, POCTOC: POC/TOC ratio, CN: carbon/nitrogen ratio, AMFCol: arbuscular mycorrhizal fungi colonization, BR: basal respiration, SIR: substrate-induced respiration, MBC: microbial biomass carbon, Nmin: mineral nitrogen.

With respect to maize shoot macronutrients, the differences between treatments were poor (Figure 4b). In fact, treatments only explained 15% of the variation in maize macronutrients (N, P, K, Ca, Mg, S). There were no differences between the rotations under MT; and under TT, R1 differed from R2 mainly due to N. Overall, TTR1 showed low levels of shoot macronutrients. Outside treatments, soil properties such as $EC_{1:2.5}$, S, Fe, Mn and Nmin explained 27% of the macronutrient variation in maize (Figure S3a). The treatment influence increased again for maize micronutrients (B, Cu, Fe, Mn, Mo and Zn) with ~36% of explained variability (Figure 4c). In general, maize after CC had a lower micronutrient concentration under MT, especially of Mn, B and Fe. Molybdenum in maize shoots increased after non-CC. Outside treatments, soil Fe and S explained 13% of the micronutrient variation in maize (Figure S3b).

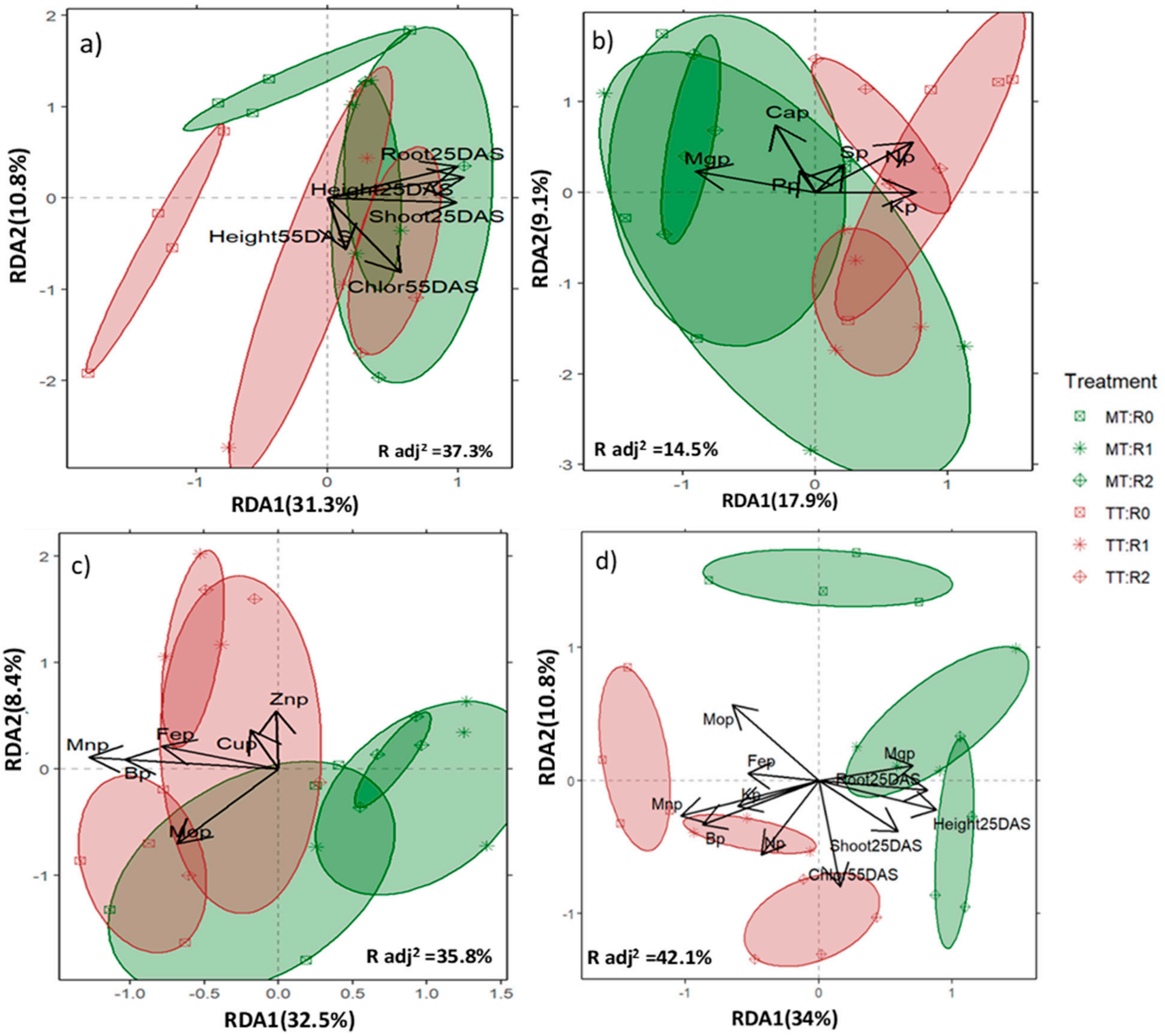

Figure 4. Redundancy analysis (RDA) results to explain: (**a**) early maize development variables, (**b**) shoot macronutrients, (**c**) shoot micronutrients, (**d**) comprehensive crop response based on 11 maize variables, from six combined treatments of tillage system (TT: traditional tillage, MT: minimum tillage) and rotation type (R0: rotation with non-CC; R1: rotation with barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 plus barley–vetch CC between wheat and maize2); DAS: days after sowing; Shoot: shoot biomass, Root: root biomass, Chlor55DAS: chlorophyll at 55 DAS maize, Xp: X nutrient concentration in shoot maize at 25 DAS maize.

We selected 11 maize variables to form part of a comprehensive crop response to treatments: four developmental (height, shoot and root biomass at 25 DAS and chlorophyll at 55 DAS), three shoot macronutrients (N, K, Mg) and four shoot micronutrients (B, Fe, Mn and Mo). Treatments explained 42% of the variation in these 11 crop variables. In view of the ellipse separation (Figure 4d), the six treatments showed quite well-differentiated crop responses. Only the two rotations with CC (R1 and R2) under MT showed a relatively similar response. The rotations tended to be ordered by the level of legume CC input within each tillage system. Outside treatments, soil properties were not significant to explain the crop response based on these 11 variables.

4. Discussion

The tillage system, with its corresponding CC termination method, modulated the soil and early crop response to different levels of legume CC input in this Mediterranean irrigated wheat-maize rotation. Thus, after only one cycle, we found very different patterns of response to the factors for different groups of soil variables (physicochemical, microbial and nutritional) and crop variables (development, macro- and micronutrients, toxic elements). All these responses were evident despite the spatial heterogeneity present in the soil. The entry of two vetch crops instead of one in the rotation and their combination with the reduced tillage proved to be the best option for early crop development, but not for soil health, as discussed below.

4.1. Soil Response to Treatments

The tillage system controlled the soil response to a greater extent than the legume CC input level, most likely due to the longer application of the tillage factor. Overall, MT was better than TT from a global soil perspective, but for a substantial number of variables, this positive effect was conditioned to the legume CC input.

Organic matter (OM) variables in topsoil benefited from reduced tillage irrespective of the CC use or not. Thus, reduced tillage increased TOC by 40% in the topsoil and almost doubled POC compared to TT, in line with several studies in Mediterranean agroecosystems [49,50]. In contrast, the weak response of OM variables to increasing inputs of fresh residues into the system may be due to the lower capacity of legume CC to build C stocks in the soil even after years [16], also to the reduced time since CC introduction, or to the initial soil spatial variability [51]. Variables such as POC, POC/TOC and even MAOC, being always higher under MT, tended to be more influenced by the tillage system in R1, so that under MT showed a maximum value, while in TT, a minimum. Under MT, OM and its fractions tended to be higher in R1 relative to R0 because of the input of extra organic residues from the CC mixture [15]. However, R2 relative to R1 involved a previous vetch entry with high quality residue (low C/N ratio) that could have had a priming effect stimulating the decomposition of previous OM depositions [52,53], whereas R1 with higher C/N residues due to barley would preserve higher values of OM variables [54]. This would explain the trend toward lower TOC, POC and even MAOC values in R2 relative to R1 under MT. In contrast, the pattern was the opposite under TT. Inverse tillage would have shifted the priming effect to R1 relative to R0 because of the CC residue incorporation, the aggregate breakdown together with the high temperature and water availability [55], so that BR and SIR were increased by 36 and 55%, respectively, in R1 compared to R0 (Figure 2). However, in TTR2, the higher residue biomass input, with higher MBC but lower BR relative to TTR1 (Figure 2), could lead to a higher microbial growth efficiency and OM stabilization [56], explaining the cause for the slightly higher values of POC (and other OM variables) in TTR2 relative to TTR1.

In this alkaline soil, pH decreased in MT with increasing OM and corresponding acid release [57], while it increased with TT as the inversion caused by the moldboard brought out deeper, more carbonate-rich soil. The EC increase in topsoil with the reduction in tillage was also reported in the same area [58] and was enhanced by CC input (Figure 2), which in this context indicates an increase in nutrient richness [59]. This nutrient enrichment in topsoil derived from the mineralization of surface crop residues, CC root exudates and reduced soil mixing compared to TT [60]. Soil bulk density was slightly higher (7% more) in MT than in TT despite the higher organic matter content in ML. This increase in bulk density is quite common when there is a reduction in tillage [61]. It is noteworthy that BD was lower in R2 than in R0 with intermediate values in R1 ($p < 0.1$). The rapid effect of rotation type on a variable such as bulk density is surprising, but on the other hand, it confirms that the use of CC can mitigate soil consolidation, resulting from reduced tillage [62]. Overall, physicochemical soil response to the treatments (56% of variance explained) was mainly driven by the tillage system, with TT equalizing soil response regardless of rotation type, while under MT, it was modulated by the higher or lower input of CC residues.

As expected, soil microbial variables were more sensitive than physicochemical variables to the entry of CCs into the system [26,63] and its termination method [30]. MBC responded to the rotation type independently of the tillage system, increasing by 23% with CC compared to non-CC. The above- and belowground organic residues from CC would increase the substrate availability for soil microorganisms, promoting its growth and hence its biomass. In their meta-analysis, Muhammad et al. [63] did not find differences in MBC between incorporation or surface disposal, but in this study, leaving CC residues on the surface and applying vertical tillage stimulated microbial biomass to a greater extent than residue incorporation, possibly due to the reduced temperature and water conservation provided by the residue mulch in this warm Mediterranean environment [62,64]. BR was strongly affected by the interaction between residue management/tillage system and legume CC input level. BR was the highest at MTR0, as concurrent with minimum Nmin and maximum soil C/N, but was the lowest at TTR0. Compared to TTR0, in MTR0, the tillage reduction stimulated the microorganism abundance (higher MBC; Table 1) [26], which immobilized N (Figure S1) and reduced the available soil N (Figure 2); moreover, the lower soil mixture and the abundance of high C/N residues (only maize and wheat) in topsoil stimulated microbial respiration (Figure 2) [26] to a larger extent than microbial growth (a higher BR to MBC ratio) because of the lower soil N, resulting in competition with the crop for soil N and the lowest maize chlorophyll value at early crop stages [65]. Thus, our study confirms the importance of combining N-fixing CCs, such as vetch, with grass main crops under reduced tillage for both soil health and yield [25].

Mycorrhizal colonization also showed a strong interaction between the two factors. TT with the incorporation of residues favored colonization, but only in R0 and R1 with a maximum in R1. The incorporation of CC residues can stimulate colonization [63], but in our case, the high AMF colonization coincided with a lower soil nutrient availability (N, P and other micronutrients), partly related to the high soil Ca values. These less favorable conditions for the plant may have stimulated root colonization by AMF [66], especially in TTR1. However, an additional vetch input in the previous year (TTR2) decreased colonization by 33% relative to TTR0 and by 42% relative to TTR1. Other studies found that legume CC did not increase subsequent maize colonization compared to non-CC [31,67], as confirmed by the meta-analysis of Muhammad et al. [63], but in those cases, the values did not reach below non-CC. The negative effect found here can be explained by the higher N input to the soil that occurs when legume residues are incorporated [68]. Mbuthia et al. [24] reported that many of the responses associated with high N fertilization [69] were observed for legume CCs such as vetch, and both cases with a detriment to mycorrhizal colonization. Very different and also related to soil N was the response under MT, where a single entry of vetch (in mixture) showed the lowest colonization value coinciding with the maximum soil N value, while a previous entry of vetch boosted colonization coinciding with a lower N value [69].

The lengths of extraradical hyphae were favored by MT irrespective of rotation type with a 40% increase compared to TT. The lower soil disturbance together with the reduced temperature and water conservation provided by residue mulching in plots under MT favored this mycelial development [70]. Similar to AMF colonization, mycelium length seemed to be penalized by a second legume CC entry in the rotation and showed its highest value with R1, so that the MTR1 combination showed 50% more mycelium length than the two combinations with less mycelium, TTR0 and TTR2.

As previously discussed, the response of the N variables was also complex. Despite having a double entry of vetch in the rotation, R2 did not increase total N, N min or MBN in soil. In their meta-analysis, Shackelford et al. [71] did not find that legume CC resulted in higher N or Nmin values in soil. The high temperatures with water availability and alkaline pH may have accelerated the mineralization of high-quality vetch residues [72], favoring N uptake by subsequent wheat or maize; in fact, maize plants in R2 showed a higher chlorophyll value relative to R0 in our sampling. The highest N and, to a lesser extent, Nmin values were found in the combination of R1 with MT and seem to be associated with a higher OM accumulation as reflected by the higher TOC and POC values. This treatment also showed the highest values of other soil nutrients such as K, Cu, Zn as well as $EC_{1:2.5}$. It cannot be ruled out that the higher growth of maize in R2 with a higher nutrient extraction may have reduced the soil nutrient concentration in R2 compared to R1. The nutrient response to treatments was relatively poor (25%), probably obscured by the soil spatial variability and periodical fertilization, but in general enhanced under MT [24].

Overall, and contrary to our hypotheses, increased legume input in the rotation did not translate into improved soil health even when combined with reduced tillage. By contrast, the combination of R1, i.e., a single input of vetch mixed with barley, with reduced tillage and residues on surface showed for many soil variables (OM variables, microbial variables and most nutrients) the better values.

4.2. Crop Response to Treatments

In contrast to soil variables, early maize development variables showed a clear response to the type of rotation regardless of tillage type. In general, both height and biomass of maize increased with the use of CC and the entry of legumes in the agroecosystem. It agrees with several meta-analyses reporting benefits in maize production after legume CC in the absence of N fertilization [73] and generally relate to improvements in soil quality, weed control and nutrient release after decomposition [74]. Although other studies reported a greater benefit from CC at minimum tillage [25], in this study, the response to rotation type was more pronounced for biomass in TT, which may be attributed to the increased mineralization of plant residues with a consequent release of N and other nutrients in inorganic form [68]. The treatments without CC showed a more differentiated behavior, more sensitive to the tillage system due to N immobilization, as explained above (Figure 4a).

With respect to maize nutrition, the treatments showed relatively few differences and, overall, the distinction by tillage system prevailed, with some modulation by the rotation type under TT. Treatment TTR1, which showed low soil macronutrient values (Figure 3c), also showed low plant macronutrient values (Figure 4b); however, with low values of micronutrients and other elements in soil, TTR1 increased its shoot concentration, even above MTR1, which had higher values in soil. This is the case of micronutrients such as Fe, Mn, Zn and toxic elements such as Pb (Figure 2 and Figure S1). This behavior of TTR1 can be attributed to the high mycorrhizal colonization, which led to a high uptake of these elements in this alkaline soil. When data of maize yield at harvest (Dr. Gabriel, personal communication) were included in the analysis, AMF colonization at 25 DAS was positively correlated with maize yield under TT ($r = 0.74$ **), highlighting its positive role in crop productivity. It should be noted that MTR1 showed favorable values for many soil variables, but this did not translate into better growth. Then, better overall soil health did not lead to better main crop performance, at least in the short-term. Soil Cd was far below the limits of toxicity [75], but attention should be paid to the possible medium to long-term accumulation of Cd under MT and increased legume input [76].

The treatments explained an important part (42%) of the crop response when considering up to 11 variables, with a well-differentiated performance (Figure 4d). Therefore, the choice of rotation with a greater or lesser legume CC input, as well as the tillage system with its corresponding termination system can largely condition the response of the crop at early stages, with a greater or lesser capacity to respond to unfavorable circumstances that may compromise the final success of the crop.

5. Conclusions

The intensification of a biannual maize–wheat rotation using different levels of legume CC input impacted soil health and maize development at early stages, even after only one complete rotation cycle. The tillage system with its corresponding CC termination and residue management modulated the soil response, especially microbial variables, to a greater extent than the crop response. The rotation with a single legume CC input in the form of a mixture of vetch and barley (R1) was greatly affected by the tillage system, mostly driven by soil OC and N variables. Against expectations, R1 combined with MT, and leaving the CC residues on the surface improved soil health the most, whereas non-CC under TT showed the worst results. The addition to R1 of a second vetch interseeded into maize (R2) improved the early maize growth irrespective of the tillage system; however, R2 combined with MT could lead to a soil OM depletion in the mid–long term. More research is needed to confirm these results in a longer term in a variety of environments to design more sustainable rotations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13051388/s1, Figure S1. Mineral-associated organic carbon (MAOC), particulate organic carbon/total organic carbon ratio (POC/TOC), electrical conductivity ($EC_{1:2.5}$), microbial biomass nitrogen (MBN), soil Fe, shoot biomass 25DAS, shoot Fe and shoot Cd, as affected by legume CC input level and tillage system interaction. Figure S2. Redundancy analysis (RDA) results to explain (a) soil microbial variables except arbuscular mycorrhizal fungi colonization, (b) soil micronutrients, (c) 10 soil physicochemical and nutritional variables, from six combined treatments of tillage system (TT: traditional tillage, MT: minimum tillage) and rotation type (R0: rotation with non-CC; R1: rotation with barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 plus barley–vetch CC between wheat and maize2). Figure S3. Redundancy analysis (RDA) results to explain (a) shoot macronutrients, (b) shoot micronutrients, from six combined treatments of tillage system (TT: traditional tillage, MT: minimum tillage) and rotation type (R0: rotation with non-CC; R1: rotation with barley–vetch CC between wheat and maize2; R2: rotation with vetch interseeded into maize1 plus barley-d vetch CC between wheat and maize2) together with soil properties.

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Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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