Effect of Leguminous Cover Crops on Soil Biological Activity in Pots of *Citrus Unshiu* Marcovitch

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Abstract

Little is known about the effects of cover crops on soil properties in citrus orchards. To fill this gap, this work was aimed to determine the effects of leguminous cover crops on the chemical and biological properties of the soil and on the structure of the microbial community in pots of *Citrus unshiu* (Marcovitch). After amendment with cover crops, an increase in total organic C (TOC), total extractable C (TEC), and total N (TN) contents were observed irrespective of the type of soil. Substrate induced respiration (SIR), and potentially mineralisable nitrogen (PMN), tested three times in one year, were higher in soils with leguminous cover crops while no significant differences were observed in protease and deaminase activity. The effect on the chemical and biochemical properties of the soil was more evident in plots containing *Trifolium subterraneum*. No changes were observed in the microbial communities studied (α-proteobacteria, β-proteobacteria, nitrogen-fixing, and ammonia oxidizers) irrespective of the kind of cover crop or type of soil, neither were variations noted during the trial.

Key-words: cover crops, leguminous, citrus orchard, soil fertility, soil biological activity.

Introduction

Use of cover crops is a way of providing the soil with organic matter and nutrients, reducing erosion and limiting losses due to the leaching of nutrients thus preserving fertility (Dinnes et al., 2002; Logsdon et al., 2002). The use of cover crops helps to improve soil structure, improving both the water infiltration and aeration, thus reducing the risk of compaction (Sainju and Singh, 1997). Other effects produced are: increased water holding capacity, improved workability of the soil, an increased cation exchange capacity, and an increase in buffering capacity (Campiglia, 1999). Organic matter, even if it is only a minor solid component of the soil, plays an important role in overall fertility (Benedetti et al., 1998). Intrigliolo and Stagno (2001) found that even slight variations in organic matter content determine a noticeable change in the physical characteristics of the soil. Much has been written regarding the relationship between soil organic matter and the activity of the microbial biomass which is fundamental if there are to be balanced cycles of the main nutritive elements, N, P and S (Benedetti et al., 1998). Biological fertility refers, in particular, to those aspects of fertility closely connected to the presence and activity of living soil organisms. Given the complex nature of the organic residues in the soil, several species of micro-organisms are involved in transforming the organic matter. This is in-

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fluenced not only by the quantity of organic residues and the levels of microbial biomass, but also by different environmental parameters such as humidity, temperature and aeration. If biological fertility is absent then the soil is sterile and can no longer guarantee the correct growth conditions for plants (Nannipieri et al., 2005).

In recent years, different methods have been developed for analysing structure and diversity of soil microbial communities. These are divided into culture-dependent and culture-independent methods, and have been used to study microbial communities in agricultural soils in temperate climates. The use of molecular markers, such as the 16S rRNA gene, has been commonly applied to explore microbial diversity in environmental samples detecting, identifying and fingerprinting micro-organisms and microbial communities (Muyzer and Smalla, 1998).

In Mediterranean environments annual self-seeding legumes are the most suitable for inclusion in agricultural systems (Caporali and Campiglia, 1993). These species correspond fully to the principles of sustainability, in that they are able to exploit, add, conserve and recycle nutritive elements, control pathogens and weeds and improve the physical characteristics of the soil (Campiglia, 1999). Autumn and winter cover crops can be very important because, given that in this period plant roots begin to absorb less nutritive elements, in this way it is possible to retain a good quantity of elements which would otherwise be lost, washed deep into the ground by the particularly intense rainfall in these seasons. At the same time, they make it possible to mobilise the generally inaccessible reserves of elements such as phosphorus. This study evaluates the effect that legume crops sown in a citrus fruit orchard have on the biological activity of the soil, measured using the following analytical parameters: substrate induced respiration (SIR), potentially mineralisable nitrogen (PMN), proteasic and deaminasic activity and bacterial community composition (Amplified Ribosomal DNA Restriction Analysis and Denaturing Gradient Gel Electrophoresis). Moreover, the effect on the total organic C (TOC), total extractable C (TEC), humic substances (HS) and total N (TN) contents was evaluated after the crops had been kept in place for two years with biomass being incorporated into the soil.

Materials and methods

Experimental set-up

The research was carried out at the S. Salvatore experimental orchard (Acireale, Catania, Italy) belonging to the Citrus Fruit Cultivation Experimental Institute, using 8-year-old satsuma plants (*Citrus unshiu* Marcovitch) grafted onto bitter oranges (*C. aurantium* L.) in 300 litre capacity plastic pots. The pots were placed in the open air and drip-irrigated. The volume of water provided was 1.5 m^3/plant/year. Mean annual rainfall was 300 mm. No fertilizer was used. The experimental design was a randomised complete block design with a 3 x 3 factorial arrangement of cover crop and soil type. The cover treatments were: a) *Trifolium subterraneum*; b) *Medicago ciliaris*; c) natural vegetation (control). The main weed species grown in the control pots were: *Bromus* spp., *Calendula arvensis*, *Chrysanthemum segetum*, *Lolium rigidum*, *Poa annua*. The soil types were: a) sandy soil (S); b) sandy-clay-loam soil (SCL); c) sandy-loam soil (SL). The treatments were repeated three times on 27 plots. During the first year of the experiment (2003-2004), both the legume crops were sown in September with a quantity of seeds equal to 250 seeds/m^2. At the end of each growth cycle in the second (2004-2005) and third years (2005-2006), the quantity of cover crop seeds produced was calculated according to their self-seeding capacity and was found to be 7,711 seeds/m^2 and 5,800 seeds/m^2 for the *T. subterraneum* and 17,591 and 22,833 seeds/m^2 for the *M. ciliaris* in the two respective years. At the end of the growth cycle, that is at the end of June or beginning of July, the organic residues and seeds produced were incorporated into the soil. Two operations are necessary when a herbaceous crop is added to the soil by means of green manuring: the biomass must be shredded to aid decomposition (increasing the surface area exposed to attack by micro-organisms) and then the dried biomass must be turned into the soil. All the operations were carried out manually.

Soil sampling and preparation

The soil samples were taken from the pots at a depth of 0-15 cm where the roots of the cover crops were present. After collection the soil was sieved through a 2 mm screen then stored in

field-moist conditions at 4 °C. The samples for molecular analysis were kept at a temperature of -25 °C. To determine the biological activity of the soil, the samples were taken in the following periods: a) July 2004 (before incorporating the biomass into the soil and 10 months after sowing); b) November 2004 (4 months after biomass incorporation); c) February 2005 (seven months after biomass incorporation); d) July 2005 (one year after incorporation). The molecular analysis were performed on soil samples taken in July 2004 and in July 2006 (one year after the second incorporation). The TOC, TEC, HS and total N calculations were performed on the same samples.

Determination of soil chemical and physical characteristics and microbial biomass activity

The main chemical and physical characteristics of the soil were measured using the standard methods recommended by the Soil Science Society of America (Page et al., 1982). SIR determination was performed according to Anderson and Domsch (1978). Carbon dioxide evolution rates were measured using the alkali absorption method. Twenty g (on a dry basis) aliquots of the soils added with glucose (4 mg/g dry soil) were placed in 500 ml glass jars containing a vial which in turn contained 4 ml of 1 N NaOH solution to trap the CO_2. After 6 hours the amounts of CO_2 absorbed by the traps were measured by tritating 2 ml aliquots with 0.1 N HC1 after previously adding 8 ml of barium chloride 0.75 N. To determine the PMN, 15 g (on a dry basis) moist soil samples were placed in 100 ml polyethylene bottles then incubated at 30 °C for 28 days. Mineral N concentrations in 0.5 M K_2SO_4 soil extracts (1:4 soil:extractant w/v ratio) were analysed at 0 and 28 days (Drinkwater et al., 1996). NH_4-N was determined using the indophenol-blue colorimetric method, while NO_3-N + NO_2-N was quantified after reduction of NO_3-N to NO_2-N on a Cu-Cd column, in accordance with Keeney and Nelson (1982). The PMN was calculated as the difference between NH_4-N + NO_3-N + NO_2-N before and after incubation.

Enzymatic determinations

To study micro-organism activity further we decided to evaluate the activity of two enzymes involved in nitrogenous metabolism: proteases and deaminases. The two enzymes were determined according to Kandeler (1993a, 1993b). All the data underwent ANOVA analysis and the means were compared with the Tuckey test.

Extraction of Total Community DNA and Nucleic Acid Amplification

DNA was extracted from the soil using the QIAamp DNA Stool Mini Kit following the manufacturer's instructions (Qiagen, Milan, Italy). The PCR was performed with a DNA Px2 thermocycler (Hybaid) using specific primers for the amplification of α-, β-proteobacterial, nitrogen-fixing and ammonia oxidiser bacteria 16S rDNA fragments. The reaction mixture for the amplification was: 1 μL template DNA (about 40 ng), 400 μM of dNTP, 1.5 mM $MgCl_2$, 5% (w/v) DMSO, 400 μM of the group-specific forward and reverse primers (Tab. 1) and 2.6 units of Taq DNA Polymerase in a buffered final volume of 50 μL (Invitrogen, Milan, Italy). Bovine serum albumin (BSA) (0.1 μM) was added to avoid organic compounds, co-extracted from the soil, from inhibiting amplification. The PCR conditions were: a hot start of 5 minutes at 94 °C, 35 cycles consisting of 1 minute at 94 °C, 1 minute at 56 °C (α-proteobacteria) / 61 °C (β-proteobacteria) / 68 °C (nitrogen-fixing bacteria), 2 minutes at 72 °C, followed by a final 10 minutes at 72°C. For ammonia oxidiser bacteria, a "touchdown PCR" program was used: 94 °C for 1 minute, 64 °C for 1 minute, 72 °C for 3 minutes, for 2 cycles; subsequently, other 8 cycles were performed lowering the annealing temperature by 2 °C every 2 cycles until it reached 56 °C and the final 15 cycles consisted of 94 °C for 1 minute, 54 °C for 1 minute, 72 °C for 3 minutes, followed by a final 10 minutes elongation at 72 °C. The PCR products, 5 μL sub-samples, were examined by electrophoresis on 1x TAE agarose gel (0.8% w/v) stained with ethidium bromide (0.5 μg mL^{-1}) with appropriate DNA size standards (Mass Ruler™, DNA Ladder Mix and Fermentas) to evaluate the size and approximate quantity of the amplicons generated. For α- and β-proteobacteria, the PCR products (1 μL) served as the template for a DGGE-PCR.

PCR amplification for DGGE analysis

α- and β-proteobacteria were amplified with the F984GC and R1378 primer sets using the group-

specific amplicons obtained, as described above, as template. Nitrogen-fixing and ammonia oxidiser bacteria were amplified with the following primer sets (FY1GC – Y2R) and (GCC-TO189FA, B, C – CTO654R), respectively. DNA, directly extracted from the soil, was used as template. The reaction mixture used for the amplification of 16S rDNA fragments for DGGE analysis was: 1 μL of template, 250 μM of dNTP, 1.5 mM $MgC1_2$, 0.1 μM BSA, 250 μM of forward and reverse primers (Table 1) and 2.6 units of Taq DNA Polymerase in a buffered final solution of 50 μL (Invitrogen). The PCR conditions were: 94 °C for 5 minutes followed by 30 cycles at 94 °C for 1 minute, 53 °C (α-, β-proteobacteria and nitrogen-fixing bacteria) / 57 °C (ammonia oxidisers) for 1 minute, 72 °C for 2 minutes and a final 10 minutes at 72 °C. The amplicons were analysed as described above.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The amplified 16 DNA products (400 ng) were digested separately for 3 hours with the restriction endonuclease TaqI (New England BioLabs, MA, USA) as specified by the manufacturer, but with an excess of enzyme (10 U per reaction). Restricted DNA was analysed by electrophoresis on 3.5% agarose gel at 30 V overnight and stained with ethidium bromide. Bands were detected from digital images (Polaroid, Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) by UV light gel transillumination (λ 312 nm).

Denaturing Gradient Gel Electrophoresis analysis (DGGE)

16S rDNA-DGGE was performed using the Dcode System (Universal Mutation Detection System, BIO-RAD). 300 ng of amplicons were loaded (top filling method) onto 6% polyacrylamide gel (Acrylamide/Bisacrylamide, 40%, 37.5:1, BIO-RAD) containing a denaturant gradient of 46-56% parallel to the electrophoresis direction made from urea and formamide (100% denaturant contains 7 M urea and 40% formamide). The gels were electrophoresed at a constant temperature (60 °C) and voltage (75 V) for 16 hours, followed by 2 hours coloration using SYBR Green I nucleic acid gel stain 1:1000 diluted in the running buffer (FMC Bio Products, Rockland, ME, USA). Bands were detected from digital images (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 2000) by UV light gel transillumination (λ 312 nm).

Results and discussion

The three soils were significantly different as regards texture, pH, active limestone, TN, TOC), TEC, and HS content. In particular, soil S appeared more acid, poorer in all forms of organ-

Table 1. Primer sequences used in the present study.

Primer	Sequence 5' a 3' (16S rDNA target)	References
F203α	CCGCATACGCCCTACGGGGGAAAGATTTAT (α-Proteobacteria)	Gomes et al., 2001
F948β	CGCACAAGCGGTGGATGA (β-Proteobacteria)	Gomes et al., 2001
R1494	CTACGG(T/C)TACCTTGTTACGAC (Bacteria)	Weisburg et al., 1991
F984GC	AACGCGAAGAACCTTAC (Bacteria)	Heuer et al., 1997
R1378	CGGTGTGTACAAGGCCCGGGAACG (Bacteria)	Heuer et al., 1997
Y1F	TGGCTCAGAACGAACGCTGGCGGC (Nitrogen-fixing bacteria)	Brosius et al., 1981
Y2R	CCCACTGCTGCCTCCCGTAGGAGT (Nitrogen-fixing bacteria)	Brosius et al., 1981
FY1GC	TGGCTCAGAACGAACGCTGGCGGC (Nitrogen-fixing bacteria)	Brosius et al., 1981
GC-clamp	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGG 5' attached to F984GC and FY1GC	Nübel et al., 1996
AMOXF	TGGGGRATAACGCAYCGAAAG (Ammonia oxidizers bacteria)	Smit et al., 1997
AMOXR	AGACTCCGATCCGGACTACG (Ammonia oxidizers bacteria)	Smit et al., 1997
GCCTO189FA	CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGA AAAGCAGGGGATCG (Ammonia oxidizers bacteria)	Kowalchuk et al., 1997
GCCTO189FB	CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGGA AAGCAGGGGATCG (Ammonia oxidizers bacteria)	Kowalchuk et al., 1997
GCCTO189FC	CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGGA AAGTAGGGGATCG (Ammonia oxidizers bacteria)	Kowalchuk et al., 1997
CTO654R	CTAGCYTTGTAGTTTCAAACGC (Ammonia oxidizers bacteria)	Kowalchuk et al., 1997

Table 2. Selected chemical and physical properties of the soils.

Soil property	S	SCL	SL
Clay (%)	2.4 a	21.2 c	11.3b
Silt (%)	3.3 a	21.8 c	13.9 b
Sand (%)	94.3 c	57.0	74.8 b
pH (1:2.5 soil KCl)	5.8 a	7.4 ab	7.6 b
pH (1:2.5 soil:H_2O)	6.5 a	8.2 ab	8.5 b
Active limestone (%)	0.37a	6.44b	14.58c
Total N (TN, ‰)	0.91a	1.88 c	1.44 b
Total organic carbon (TOC, %)	0.99 a	1.79 b	1.19 a
Extractable organic carbon (TEC, %)	0.66 a	1.10 b	0.80 a
Humic substances (HS, %)	0.48 a	0.74 b	0.54 ab

S = sandy soil; SCL = sandy-clay-loam soil; SL = sandy-loam soil.
Standard deviation < 10%. Values followed by the same letters within the grouping of rows are not significantly different by the F-test in the analysis of variance ($p < 0.05$); n = 9).

ic matter and TN. Instead, the P content of the soil S was higher than the other two soils. Soil SCL was the richest in TOC, TEC, HS and TN. Soil SL contained the greatest quantity of active limestone and was consequently characterised by the highest pH (Tab. 2). Among the parameters considered in evaluating the chemical characteristics of the three soils, TOC, TEC, HS and TN content were measured both in July 2004 (before the first incorporation of biomass) and in July 2006 (before the third incorporation of biomass). Table 3 compares the relative percentage values of the samples taken in July 2006 compared with those of July 2004. An increase in TOC, TEC and TN can be seen in all the experimental conditions while the HS content was higher in the lots containing *T. subterraneum*, practically unchanged in the lots with *M. ciliaris* and 16% lower in the lots with spontaneous vegetation. As regards the TOC and TN the increases appear greater in the lots containing legumes, but only in the case of *T. subterraneum* was a significant difference seen as compared with the control. The increase in TEC was comparable in all three experimental conditions. Data collected from commercial dryland and irrigated legume crops indicated that 95-270 kg fixed N ha^{-1} can remain in vegetative residues after harvesting (Peoples et al., 1995; Rochester et al., 1998; Schwenke et al., 1998). Spehn et al. (2002) found that the presence of legumes enhanced nitrogen availability for co-occurring species, which increased their biomass production and tissue nitrogen concentrations. The fixed nitrogen was made available to non-fixing neighboring plants by the mineralization of legume litter (Peoples and Crashwell, 1992). Comparing the trend of the parameters no relevant differences were observed with respect to the type of soil, even if in soil SL the increases appeared to be generally lower, probably due to its higher pH.

The evaluation of the biological activity was performed only over a brief period with three measurements being made in the course of one year. The results of the analyses are shown in Table 4. The SIR was higher in those lots containing legumes as compared to the control, but the difference was only statistically significant in the case of *T. subterraneum*. The N-NH_3 con-

Table 3. Effects of amendment with cover crops on selected soil chemical characteristics. Comparison between the soil samples collected on July 2004 and July 2006.

Soil property	Comparison between cover crops			Comparison between soils		
	T. subterraneum (%)	*M. ciliaris*	Control	S	SCL	SL
Total N	140	126	119	138	134	120
TOC	130	124	121	120	135	120
TEC	120	117	119	120	120	116
HS	138	97	84	102	106	111

S = sandy soil; SCL = sandy-clay-loam soil; SL = sandy-loam soil.
Standard deviation < 10%; (n = 9).

Table 4. Biological properties of the soils amended with cover crops.

Soil property	Comparison between cover crops			Comparison between soils		
	T. subterraneum	*M. ciliaris*	Control	S	SCL	SL
SIR (μg C-CO_2)	95.9 b	78.3 ab	63.0 a	23,4 a	103. c	76. b
PMN (μg g^{-1})	17.6 a	18.5 a	24.5 b	14.8 a	23.9 b	22.3 b
N-NO_3 (μg g^{-1})	16.3 ab	18.3 b	12.3 a	11.9 a	22.3 b	13.1 a
N-NH_4 (μg g^{-1})	7.7 a	8.1 a	7.6 a	8.9 a	8.4 a	6.3 a
Proteases (μg tiroxine g^{-1})	99.0 a	117.2 a	102.2 a	128.0 b	92.2°	98.2 ab
Deaminases (μg N g^{-1})	14.7 a	12.8 a	14.8 a	15.6 a	15.6 a	10.9 a

S = sandy soil; SCL = sandy-clay-loam soil; SL = sandy-loam soil.
Standard deviation < 10%. Within each comparison, rows means followed by the same letters are not significantly different by the F-test in the analysis of variance ($p < 0.05$); ($n = 9$).

tent was also greater in the lots planted with legumes but, in this case, only those containing *M. ciliaris* presented a significantly higher content than the control. The greater N-NH_3 content could be due to rhizobium activity which is normally in symbiosis with this vegetable species. The N-NH_4 content was more or less equal in all three experimental situations. In the lots containing legumes microbial activity, responsible for the mineralization of organic N, was slightly inhibited. In fact, the PMN values were lower as compared to the control. This could be attributed to a greater quantity of N-NH_3 coming from the fixing of atmospheric N by the rhizobia. Finally, the different vegetation cover did not seem to have influenced enzymatic activity. Considerable differences were found in the biological parameters of the different soils. Soils SCL and SL had a more intense microbiological activity expressed by the higher values of SIR and PMN. Nonetheless, the enzymatic activity was either comparable in the three soils (deaminases) or higher in soil S as compared to soils SCL and SL (proteases). Positive correlations were found between the SIR and TOC ($R^2 = 0.75$), TEC ($R^2 = 0.82$), total N ($R^2 = 0.97$) and PMN ($R^2 = 0.99$). These correlations indicate, on the one hand, that the microbial population was more active in the soils containing the necessary nutrients for its growth (in particular N which often constitutes a limiting factor) and, on the other, that the activity of the ammonifying and nitrifying bacteria proceeded at the same rate as the total microbial activity.

Molecular analyses

In July 2004 and July 2006 different bacteria groups, the α-proteobacteria, β-proteobacteria, nitrogen-fixing bacteria and ammonia oxidisers,

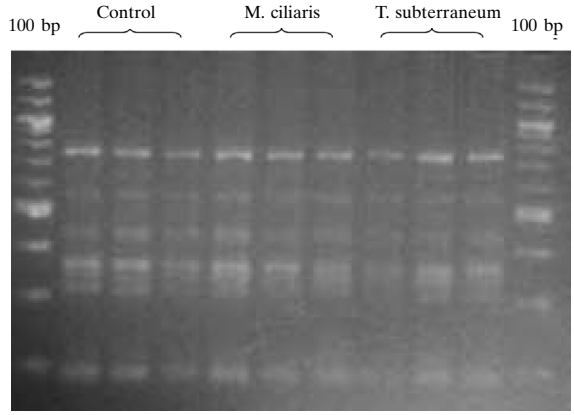

Figure 1. ARDRA electrophoretic profiles of 16S rDNA of α-proteobcteria. Soils sampled on July 2004.

were isolated from soil samples of citrus plants and analysed using molecular techniques. Figure 1 shows the electrophoretic profiles of the α-proteobacteria obtained using the ARDRA technique. No significant differences were found in the samples, the restriction enzyme Taq I generated an electrophoretic pattern of 7 bands of equal intensity, in which the fragments obtained are spread between about 750 and 180 bp. These results were confirmed by DGGE analyses (Fig. 2a). The same results were also obtained for the samples taken in July 2006, where the DGGE analysis produced electrophoretic patterns which were identical both in terms of number and band intensity (Fig. 2b). The β-proteobacteria and the nitrogen-fixing bacteria, and the ammonia oxidisers also produced similar results (data not shown). No significant differences were seen in the electrophoretic ARDRA and DGGE patterns in the various tests: the gels showed an equal

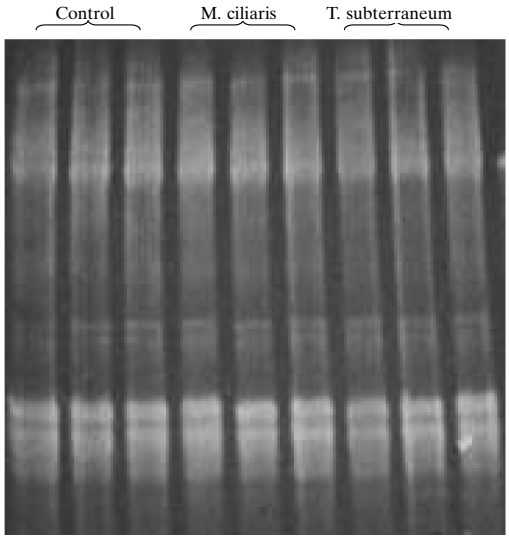

Figure 2. DGGE banding pattern of 16S rDNA of α-proteobacteria. Soils sampled on July 2004 (a) and July 2006 (b).

number of bands of equal intensities confirming that the samples were very similar. This result was obtained with the samples collected both in July 2004 and July 2006. Only the results relating to the sandy-loam soil (SL) are reported because those relating to the sandy soil (S) and the sandy-clay-loam soil (SCL) revealed a similar trend. Our results agree with that of Peixoto et al. (2006) who determined the effects of conventional tillage and no-tillage in crop systems with and without cover crops on bacterial structure. They reported differences in response to cultivation, tillage and depth, but not due to cover cropping.

Conclusion

The research made it possible to highlight that legume crops produce an increase in microbial activity (SIR) and N-NH_3 content in the soil while they inhibit the activity of micro-organisms involved in the mineralization of organic N. This last fact would seem to be correlated to the increase in N-NH_3 as a consequence of the fixing of atmospheric N by the symbiotic rhizobia. In the long term, the presence of legumes leads to an increase in TOC, TEC and total N. The increase in these parameters also occurs in areas producing spontaneous ground cover, even if to a lesser degree. This indicates that the use of cover crops brings about an improvement in soil fertility.

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