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RESEARCH ARTICLE

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Soil microbial functionality in response to the inclusion of cover crop mixtures in agricultural systems

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Abstract

Agricultural systems where monoculture prevails are characterized by fertility losses and reduced contribution to ecosystem services. Including cover crops (CC) as part of an agricultural system is a promising choice in sustainable intensification of those demanding systems. We evaluated soil microbial functionality in cash crops in response to the inclusion of CC by analyzing soil microbial functions at two different periods of the agricultural year (cash crop harvest and CC desiccation) during 2013 and 2014. Three plant species were used as CC: oat (*Avena sativa* L.), vetch (*Vicia sativa* L.) and radish (*Raphanus sativus* L.) which were sown in two different mixtures of species: oat and radish mix (CC1) and oat, radish and vetch mix (CC2), with soybean monoculture and soybean/corn being the cash crops. The study of community level physiological profiles showed statistical differences in respiration of specific C sources indicating an improvement of catabolic diversity in CC treatments. Soil enzyme activities were also increased with the inclusion of CC mixtures, with values of dehydrogenase activity and fluorescein diacetate hydrolysis up to 38.1% and 35.3% higher than those of the control treatment, respectively. This research evidenced that CC inclusion promotes soil biological quality through a contribution of soil organic carbon, improving the sustainability of agrosystems. The use of a CC mixture of three plant species including the legume vetch increased soil biological processes and catabolic diversity, with no adverse effects on cash crop grain yield.

Additional key words: microorganisms; soil functionality; sustainability; diversification; enzymes**.**

Abbreviations used: AS (aggregate stability); AUC (area under curve); BD (bulk density); CC (cover crop); CC1 (oat and radish mix); CC2 (oat, radish and vetch mix); CLPP (community level physiological profile); DHA (dehydrogenase activity); DM (dry matter); FDA (fluorescein diacetate); INT (iodonitrotetrazolium); PCA (principal component analysis); RFU (relative fluorescence units); SOC (soil organic carbon).

Authors' contributions: Conceived and designed the experiments: SBR, AEA and SVG. Supervised the work: JMM and SVG. Performed the experiments: DNC, RAV, EJM and CC. Analyzed the data: DNC, EJM and CC. Contributed reagents/materials/analysis tools: RAV, SBR, AEA and JMM. Wrote the paper: DNC.

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Introduction

Soybean production area in Argentina has increased from 0.01 million to more than 27 million hectares in the last 30 years, making Argentina the world's third largest producer (Sinclair *et al*., 2007). This increase in soybean cropping frequency in the agricultural systems, mostly once a year, leads to an inefficient use of resources (*i.e.* water and solar radiation) during the fallow period, thus dramatically reducing the efficiency and productivity of the system (Novelli *et al*., 2011). The loss of fertility recorded in soils cultivated predominantly with soybean is mainly due to the low annual contribution of organic carbon (C) to the soil

and long fallow periods during autumn/winter (Restovich *et al*., 2011; Moharana *et al*., 2012; Kintché *et al*., 2015). In this scenario, it is necessary to diversify agricultural systems and preserve soil health and therefore sustain productivity and profitability. The inclusion of cover crops (CC) is a promising option aimed at sustainable production of agricultural systems. The type and characteristics of these crops (*e.g*., root depth, release of nutrients in the rhizosphere, adaptation to climatic conditions) are key factors related to improving soil biological, chemical and physical conditions (Constantin *et al*., 2010; Blanco-Canqui *et al*., 2011; Sapkota *et al*., 2012).

Microbial communities are highly sensitive and respond rapidly to changes. Therefore, monitoring their variation is a valuable tool to provide early signals of altered soil environment (Bastida *et al*., 2008). A number of soil microbial variables, such as respiration, enzyme activities and community level physiological profiles, have the potential for being used as indicators of soil functionality in response to crop management (Pérez-Brandán *et al*., 2014). Accordingly, management practices such as the use of cover cropping can substantially alter labile soil C and soil moisture, which may have unique impacts on microbial community structure (Buyer *et al*., 2010). However, little is known about the effect of CC inclusion on soil biological processes. Since plant species differ in their biochemical composition, changes in plant diversity are likely to alter the quantity and quality of soil chemical resources, thereby controlling the composition and consequently the functioning of soil microbial communities (Nilsson *et al*., 2008). However, there are gaps of knowledge concerning the effect of the inclusion of certain CC species on soil biological functioning: which species could improve soil processes? Is it viable to seed them in mixtures to produce a synergic effect? Which mixture would better improve soil functioning?

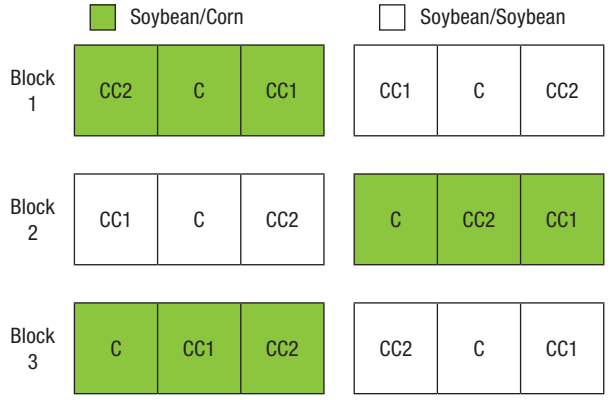
Understanding the interactions between members of the microbial community is fundamental to elucidate the ecosystem functioning. However, the methodological techniques used to describe the structural composition of communities cannot be used for that purpose since a diverse microbial community does not necessarily mean a major microbial activity or reflect the biological processes occurring in living cells (Deng *et al*., 2012; Rao *et al*., 2014). This study is focused on the functioning of soil microbial communities as a result of agrosystem diversification evaluated at two different periods of the agricultural year: cash crop harvest (soybean monoculture and soybean/corn rotation) and CC desiccation, to meet the following objectives: 1) to evaluate the short-term response of soil microbial functionality on the inclusion of different CC families (Brassicaceae, Leguminosae, Gramineae), seeded as part of two different mixtures of species, 2) to determine the interaction between microbiological soil variables and soil organic C in response to the inclusion of CC, and 3) to assess soybean productivity in response to the diversification of the agricultural system. We hypothesized that diversification of extensive agricultural systems through the inclusion of CC mixtures improves soil functionality and catabolic diversity in the short-term, and this improvement varies according with the CC used through the amount of soil organic C added to the soil by CC inclusion.

Material and methods

Field experiment

This study was conducted at the Pergamino Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA) (33°51′S, 60°40′W), Buenos Aires province, Argentina, in 2013 and 2014. The soil in the study area is predominantly Typic Argiudoll (USDA Soil Taxonomy) of the Pergamino series with a silt loam A horizon without eroded phase (<0.3% slope). The climate is temperate humid, with mean annual temperature of 16.5°C (Hall *et al*., 1992) and mean annual rainfall of 971 mm for the 1910–2010 period (Agroclimatological Network Database, INTA; http://climayagua.inta.gob.ar/). Rainfall and drainage occur mainly in autumn and spring and the summer months usually present water deficits of varying intensity (Hall *et al*., 1992). The field trial under no-tillage was started in March 2011 and soil sampling was performed during 2013 and 2014. The experimental design consisted of a split plot design in a randomized complete block arrangement (Fig. 1). The main plot (30 m long × 15 m wide) corresponded to the cash crop sequence (soybean/soybean and soybean/corn) and was divided into three subplots (30 m long × 5 m wide), that represented the CC and the control. Species used as CC were: oat (*Avena sativa* L.), vetch (*Vicia sativa* L.) and radish (*Raphanus sativus* L.), being sown in two different mixtures of species: oat and radish mix (CC1) and oat, radish and vetch mix (CC2), including a control treatment without CC. The experiment consisted of six treatments with three replicates each, in three blocks, totaling 18 subplots and six main plots. The treatments were: a) soybean/soybean CC1; b) soybean/soybean CC2; c) soybean/soybean control; d) soybean/corn CC1; e) soybean/corn CC2; and f) soybean/corn control.

Soybean (*Glycine max* L.) hybrid DM5.1 (Don Mario) was sown in rows spaced 0.52 m (500,000 plants/ha) and corn (*Zea mays* L.) hybrid DK 747



Soil sampling

2013		2014	
March	**August**	**March**	**August**
Soybean and corn harvest	CC desiccation	Soybean harvest	CC desiccation

Figure 1. Field experiment scheme and soil sampling periods. CC2: oat, radish and vetch mix. CC1: oat and radish mix. C: control. CC: cover crops.

(Monsanto) was sown in rows spaced 0.70 m (75,000 plants/ha). Both cash crops were sown by direct drilling. Corn was fertilized at seeding with calcium superphosphate (150 kg/ha) and at V5-6 with 32 kg N/ha. CC were sown under no-tillage immediately after cash crop harvest (soybean or corn) and fertilized at seeding with 14.7 kg of P_2O_5/ha. Vetch was inoculated before sowing with *Rhizobium leguminosarum* biovar. *Vicea*. The soybean and corn sowing dates and the distribution of rainfall determined the end of the CC growing periods. Before planting corn, CC were desiccated in winter or early spring (August-September), during their vegetative stage. Before planting soybean, CC were desiccated in spring (October) during their reproductive stage. CC were desiccated with 3- 4 l/ha of glyphosate (48% active ingredient) and their residues were left in the surface soil, without tilling them into the soil.

Soil sampling

Soil sampling was performed twice a year in 2013 and 2014, with the first sampling being conducted at cash crop harvest (Fig. 1). The second sampling was performed at CC desiccation. The sampling methodology followed a previous report (Restovich *et al*., 2012). Six composite soil samples were taken per plot from the horizon A, at a depth of 10 cm, from six sampling stations. Soil samples were passed through a 2-mm sieve and stored at 4ºC for one day prior laboratory analysis.

Community-level physiological profiles (CLPP)

Functional microbial diversity was quantified using community-level physiological profiles (Ruiz *et al*., 2008). The selected carbon sources used comprised a range of quality and complexity of substrates which consisted of six monosaccharides (D-dextrose, D-mannose, D-fructose, D-glucose, D-galactose, D-xylose) one disaccharide (D-lactose), four amino acids (DL-tryptophan, L-arginine, L-asparagine, L-lysine), and one vitamin (thiamine). Briefly, stock solution was prepared with each carbon source in deionized water (3 g/L), filter-sterilized and stored at 4 ºC in the dark. A basal medium consisted of K_2HPO_4 (21 g/L), KH_2PO_4 (9 g/L), $MgSO_4$ (0.3 g/L), $(NH_4)_2SO_4$ (1.5 g/L), $CaCl_2$ (0.03 g/L), $FeSO_4$ (0.015 g/L), $MnSO_4$ (0.0075 g/L), $NaMoO_4$ (0.0075 g/L). A portion (5 g) of each soil sample was suspended in 10 mL of filter-sterilized deionized water. Each individual microplate was prepared with stock solution (60 Wl), basal medium (60 Wl), and tretrazolium violet (0.0075%). Finally, soil suspensions (120 Wl) were added into the wells, and plates were immediately incubated at 25ºC. Readings were obtained from the plate at 24, 48 and 72 h (Wallac 1420 Victor2 multi-label counter, Perkin Elmer Life Sciences). Readings at each time point (relative fluorescence units, RFU) were plotted *vs*. time (hours) to obtain respiratory curves (Allegrini *et al*., 2015). The integrated area under respiratory curve (AUC) was calculated between 24 and 72 h with the software SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

Microbial respiration

In order to measure soil microbial respiration, 10 g of moist sample were weighed in a vessel and then placed inside a stoppered glass jar. After 7 days of incubation, the CO_2 evolved was trapped in 15 mL 0.2 N NaOH placed in another vessel inside the jar. After the incubation, excess 1 M $BaCl_2$ was added to the NaOH solution for CO_3 precipitation and the remaining NaOH was titrated with 0.2 N HCl using phenolphthalein as an indicator. Blanks were used to account for carbon dioxide absorption during handling and titration (Alef, 1995).

Enzyme activities

Fluorescein diacetate hydrolysis (FDA). FDA hydrolysis was estimated according to Gillian & Duncan (2001). Briefly, 2 g of soil and 15 mL of 60 mM potassium phosphate buffer pH 7.6 were placed in a 50 ml conical flask. Substrate (FDA, 1,000 µg/

mL) was added to start the reaction. The flasks were placed in an orbital incubator at 30ºC for 20 min, 100 rpm. Then the flasks were removed from the incubator and 15 mL of chloroform/methanol (2:1 v/v) was added immediately to terminate the reaction. The contents of the conical flasks were then centrifuged at 2,000 rpm for 5 min. The supernatant was then filtered and measured at 490 nm on a spectrophotometer.

Dehydrogenase activity (DHA). DHA was determined according to García *et al*. (1997). Soil (1 g) at 60% of field capacity was exposed to 0.2 mL of 0.4% INT (2-p iodophenyl-3-pnitrophenyl-5-phenyltetrazolium chloride) in distilled water at 22ºC in darkness for 20 h. The INTF (iodonitrotetrazolium formazan) formed was extracted with 10 mL of methanol by shaking vigorously for 1 min and filtered through a Whatman N°5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Physical properties and soil organic carbon (SOC)

Soil physical properties were measured only at cash crops harvest considering their little variability over time. Aggregate stability (AS) was measured according to Douglas & Goss (1982). Briefly, 10 g of 1–2-mm diameter aggregates at field moisture were placed on a 0.5-mm sieve and mechanically raised and lowered into water for 5 min. The stability index was calculated by Kemper's (1965) procedure:

AS= [Aggregate dry weight on the sieve (<0.5mm) / Aggregate dry weight (1-2mm)] * 100

Bulk density (BD) was determined by the method of cylinder according to Burke *et al*. (1986). SOC was determined by the Walkley-Black method (Page, 1982).

Grain yield

Soybean grain yield was determinate in 2014 (year in which every main plot was under soybean crop). Grain yield was measured at physiological maturity by mechanically harvesting six rows of 10 linear meters in each main plot. Grain weights were adjusted to 13% water content. Dry pods were threshed to separate the seeds from the chaff, and weights of the seeds (grain yield) taken thereafter. The yield was expressed as the measured grain yield (kg DM) per land area (ha).

Statistical analyses

The studied variables were analyzed using linear mixed models with Fisher test (LSD) at $p<0.05$ using InfoStat-Professional (Di Rienzo *et al*., 2013). Data obtained from the two years evaluated were pooled in order to study the effect of the treatments on soil microbial variables. Fixed effects were covering (CC1, CC2 and control), cash crop sequence (soybean monoculture and soybean/corn) and their interaction (covering*sequence), whereas random effects were year and block. In all cases, residuals were tested for normality with the Shapiro-Wilks' test. CLPP data was analyzed through the AUC, which was calculated with the software SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA). Also, principal component analysis (PCA) was performed to analyze CLPP data and to study the effect of the treatments on soil microbial and physical properties, since PCA summarizes a high dimensional space and order samples on a 2-dimensional plane, while preserving the maximum allowable variance within the data set (Zhao *et al*., 2012). Finally, considering that C content has a great influence on biological soil conditions, correlations between soil biological variables and SOC were estimated using Pearson's coefficient with significance at $p \leq 0.01$ and $p \leq 0.001$.

Results

Community-level physiological profiles (CLPP)

The C sources utilization profiles are shown in Fig. 2A-B with no significant interaction between sequence and CC or significant effect of the sequence. Significant differences ($p<0.05$) were found in respiratory responses reflected by AUC between CC treatments at cash crop harvest (dextrose, mannose, fructose, glucose, xylose and tryptophan, Fig. 2A) and at CC desiccation (dextrose, mannose, fructose, glucose, lactose, xylose, galactose, tryptophan, arginine, asparagine, lysine, Fig. 2B). At cash crop harvest, the AUC corresponding to dextrose, mannose, fructose and glucose were on average 11.6% and 9.3% higher in CC2 and CC1, respectively, than in control treatment; also xylose and tryptophan were 16.7% and 30% higher in CC2 than control, respectively, being CC1 17.2% lower than CC2 for tryptophan. At CC desiccation, the AUC corresponding to dextrose, mannose, fructose, glucose, lactose, xylose, galactose and tryptophan were on average 43% and 26.5% higher in CC2 and CC1, respectively, than control treatment; besides CC1 was 24% and 43% lower than CC2 for fructose

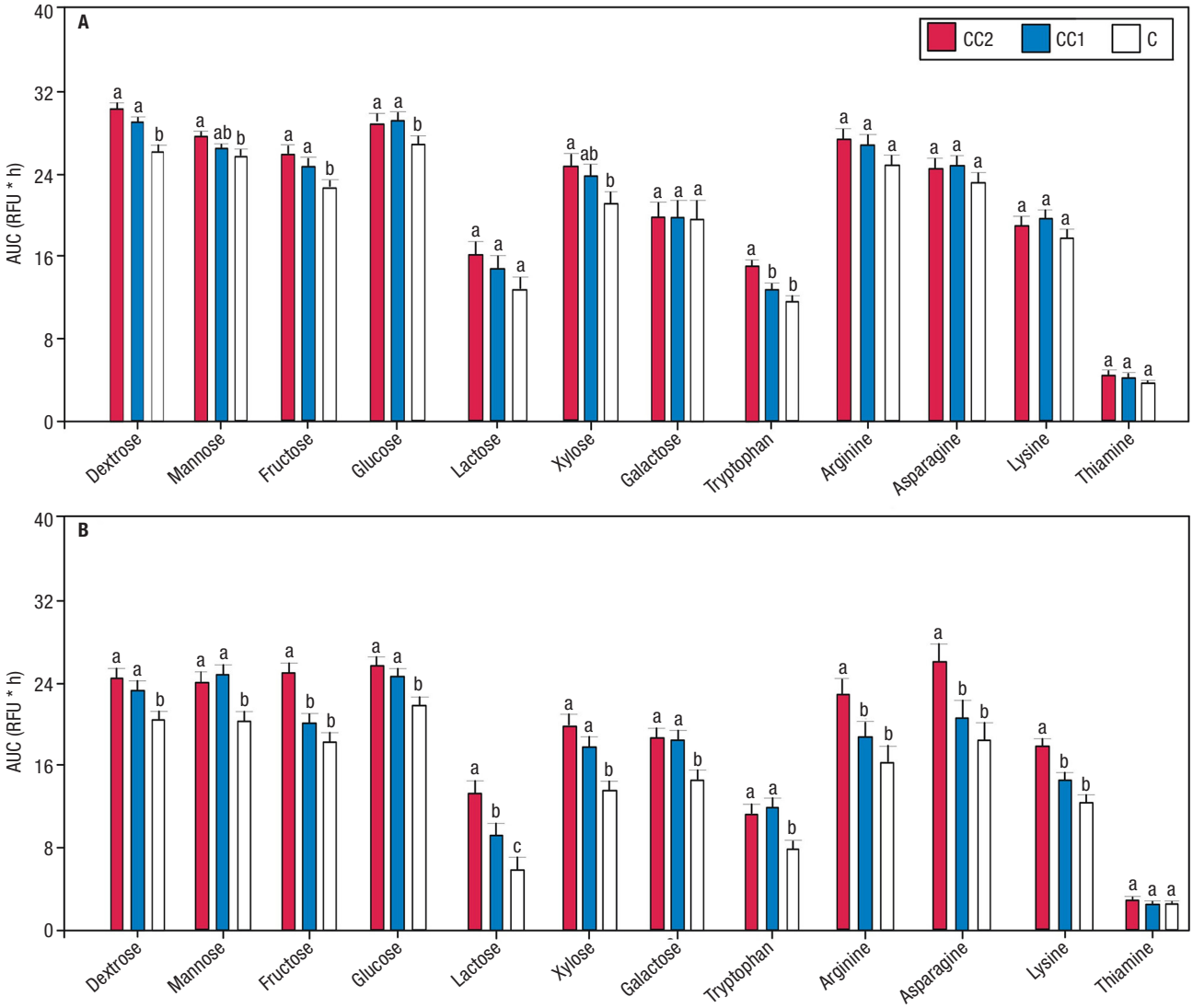


Figure 2. Community level physiological profiles (CLPP) in response to the inclusion of cover crop (CC) treatments, CC1 (oat and radish mix), CC2 (oat, radish and vetch mix) and control (C), after soybean/corn rotation and soybean monoculture, at two sampling periods, cash crop harvest (A) and CC desiccation (B), 2013-2014. The area under the respiration curve (AUC) is indicated for each C source. Different letters within each C source are significantly different (p<0.05); error bars indicate standard deviation.

and lactose, respectively. The AUC corresponding to arginine, asparagine and lysine were on average 23.3% and 41.4% higher in CC2 than in CC1 and in control treatment, respectively.

PCA was performed to study the C utilization profiles obtained after 72 h of incubation (Fig. 3). CLPP showed a general high consumption of the C substrates in the CC treatments. At cash crop harvest, PC1 and PC2 accounted for 40.9% and 11.8% variance, respectively. CC2 treatment was separated from CC1 and the control treatment along PC1 in both monoculture and soybean/corn rotation plots (Fig. 3A). A clear separation of cash crops was evidenced along PC2. An even more marked effect of the inclusion of CC on catabolic diversity was observed at CC desiccation, since at this sampling period PC1 and PC2 explained 53.3% and 8.7% of the data variance, respectively (Fig. 3B). At CC desiccation, the control treatment of soybean/corn rotation was separated from both CC treatments, which grouped together. In soybean monoculture plots, the same behavior observed at cash crop harvest was observed at CC desiccation, since CC2 was also separated from the rest of the treatments along PC1.

Soil microbial respiration

Soil microbial respiration behaved similarly at the two sampling periods (Table 1), with no significant interaction between sequence and CC or significant effect of the sequence. At cash crop harvest, a significant effect

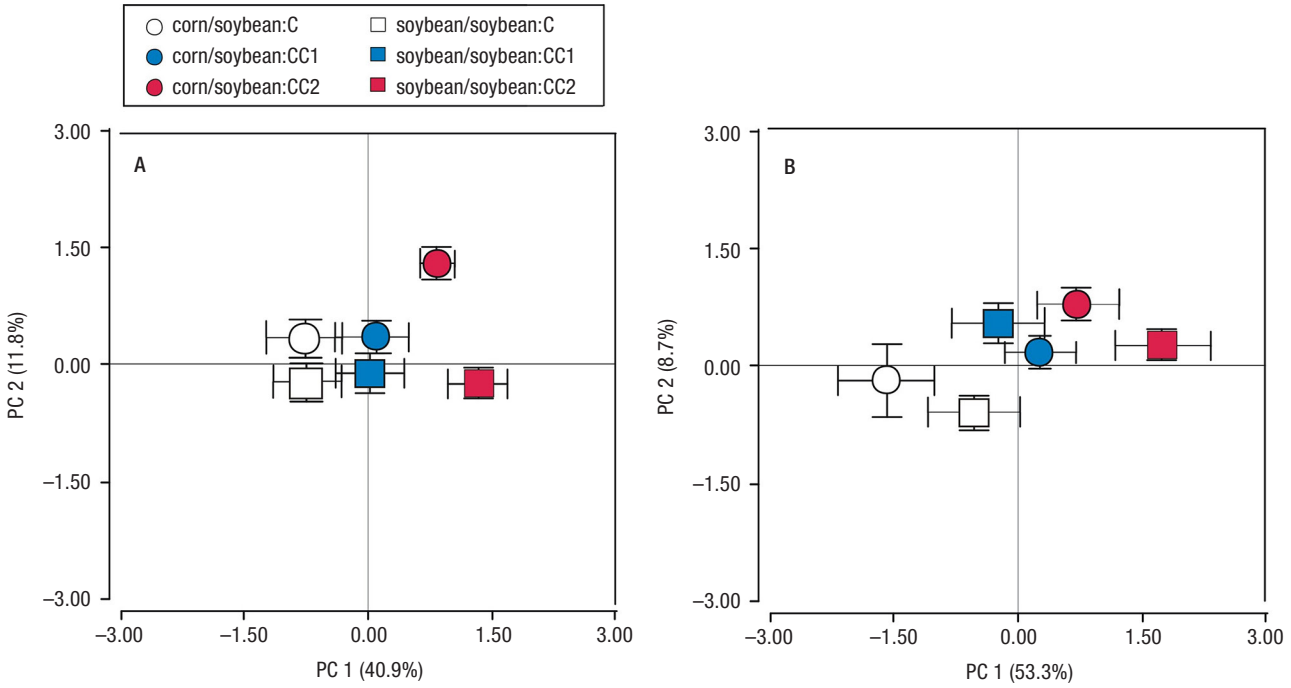


Figure 3. Principal component (PC) analysis of the community-level physiological profiles (CLPP) incubated over 72 h in response to the inclusion of cover crop (CC) treatments, CC1 (oat and radish mix), CC2 (oat, radish and vetch mix) and control (C), after soybean/corn rotation and soybean monoculture, at two sampling periods, cash crop harvest (A) and CC desiccation (B), 2013-2014.

of CC inclusion was observed, with microbial respiration being markedly affected by CC. At this sampling period, both CC2 and CC1 were significantly higher (31.4%) than the control treatment. Similarly, at CC desiccation, CC2 and CC1 were also significantly higher (45.3% and 37.7%, respectively) than the control.

Enzyme activities

No significant interaction between sequence and CC or significant effect of the sequence was observed for any enzyme activities (Table 1). FDA hydrolysis was significantly higher under the inclusion of CC at both cash crop harvest and CC desiccation. At cash crop harvest, FDA was 20.4% and 14.6% higher in CC2 and CC1 treatments, respectively, than in the control. At CC desiccation, FDA was 35.3% and 16.7% higher in CC2 and CC1 than in the control, respectively, and 15.8% higher in CC2 treatment than in CC1.

Dehydrogenase activity (DHA) showed a similar trend to FDA at cash crop harvest, showing an increase of 27.2% and 21.0%, in CC2 and CC1, respectively, with respect to control treatment. At CC desiccation, the same behaviour was observed, with DHA being 38.1% and 29.6% higher in CC2 and CC1 than in the control, respectively, with no significant differences between CC1 and CC2 for this variable.

Table 1. Soil organic carbon (SOC; g/kg), microbial respiration (MR; mg CO_2/g), fluorescein diacetate hydrolysis (FDA; g FDA/g) and dehydrogenase activity (DHA; g INTF/g) at cash crop harvest and CC desiccation, 2013-2014, under different cover crops (CC) treatments, CC1 (oat and radish mix), CC2 (oat, radish and vetch mix) and control. In each column different letters are significantly different according to LSD test ($p \leq 0.05$).

Covering	Cash crop harvest				CC desiccation			
	SOC	MR	FDA	DHA	SOC	MR	FDA	DHA
CC2	22.83 a	0.46 a	123.77 a	70.98 a	25.54 a	0.77 a	143.68 a	55.25 a
CC1	23.02 a	0.46 a	117.85 a	67.50 a	25.39 a	0.73 a	124.03 b	51.87 a
Control	21.15 b	0.35 b	102.83 b	55.78 b	21.33 b	0.53 b	106.20 c	40.04 b
***p* value**								
Covering	0.0018	<0.0001	<0.0001	0.0013	<0.0001	<0.0001	<0.0001	0.0001
Sequence	0.4883	0.0549	0.8844	0.547	0.4601	0.0537	0.1335	0.0824
Covering*Sequence	0.7771	0.1469	0.9795	0.088	0.1682	0.9553	0.089	0.9004

Soil organic C

Soil organic C (SOC) showed higher values in response to the inclusion of CC (Table 1); no significant interaction was observed between sequence and CC. At cash crop harvest, a significant effect of CC inclusion was observed, with SOC being significantly higher in CC2 (22.83 g/kg) and CC1 (23.02 g/kg) than in control treatment (21.15 g/kg). The same trend was observed at CC desiccation, when a significant effect of CC inclusion was recorded. At this sampling period, SOC was significantly higher in CC2 (25.54 g/kg) and CC1 (25.39 g/kg) than in control treatment (21.33 g/kg) as well. No significant differences were observed between the two mixtures of species used as CC for this variable.

Considering the results obtained, a correlation analysis was performed to study the correlations between microbiological variables and SOC content at cash crop harvest and CC desiccation. According to Pearson coefficients, microbial respiration, FDA hydrolysis and DHA showed a significant and positive correlation with SOC at both sampling periods (Table 2). Also, a PCA (Fig. 4) was performed to analyze the influence of the variables in the differentiation of treatments. This analysis showed the positive correlation between SOC and microbial activity variables, which were the most relevant to separate treatments along PC 1.

Soil physical proprieties and grain yield

Increased AS was observed with the inclusion of CC, whereas for BD, no significant differences were recorded (data not shown). AS was 29.7% and 49.8% higher in CC2 and CC1 treatments, respectively, than in the control, with no significant differences between CC1 and CC2 for this variable.

No statistical differences were observed in grain yield among treatments during the period studied (data not shown).

Discussion

Catabolic diversity

The present study investigated the overall effect of CC inclusion on a series of microbial properties, in-

Table 2. Correlation analysis between soil properties and soil organic carbon (SOC) at cash crop harvest and CC desiccation, 2013-2014.

Soil parameters	Pearson coefficients SOC	
	Cash crop harvest	CC desiccation
Microbial respiration (mg CO_2/g·week)	0.32 **	0.36 **
FDA hydrolysis (ig FDA/g·h)	0.63 *	0.42 **
DHA (µg INTF/g soil·h)	0.56 **	0.30 **

*,** Significant at $p < 0.01$ and $p < 0.001$, respectively.

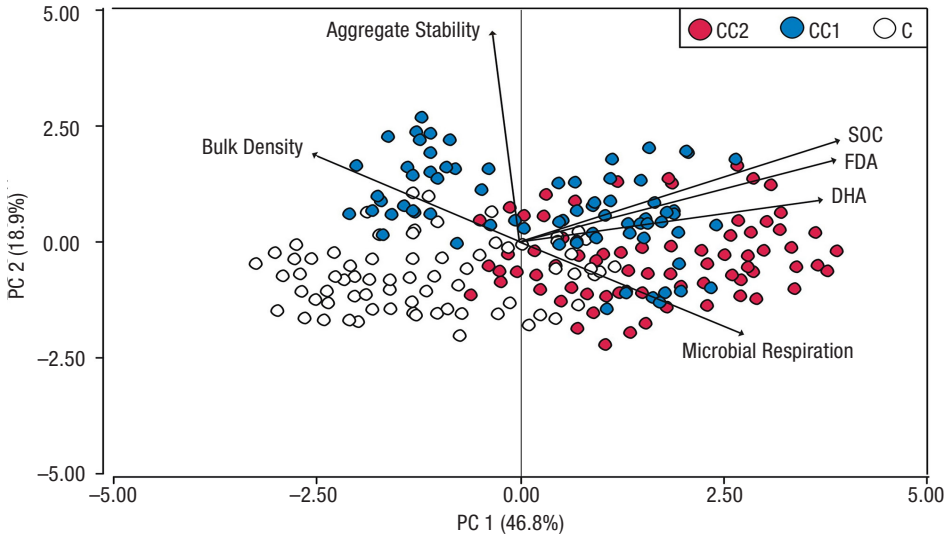


Figure 4. Principal component (PC) analysis of soil properties measured at cash crop harvest, 2013-2014, in response to the inclusion of cover crop (CC) treatments, CC1 (oat and radish mix), CC2 (oat, radish and vetch mix) and control (C). Vectors indicate the degree of correlation between each factor and the axes. FDA: fluorescein diacetate hydrolysis. DHA: dehydrogenase activity. SOC: soil organic carbon.

cluding catabolic diversity, microbial respiration and soil enzyme activities. These properties allowed us to detect changes in soil functionality between both CC mixtures studied, in order to relate those changes to SOC. The study of CLPP was performed to evaluate the variations in functional diversity and physiological profiles among the microbial communities from CC treatments. Even though CLPP can only show the changes of cultivable microbes, it can be used for analyzing microbial community physiological activity and their relations with microbial variables (Huang *et al*., 2015). In our study, the substrate utilization results reflected by AUC showed that CC induced important functional changes in the microbial community. A general increase of C sources consumption was found in CC treatments at both sampling periods. According to this, Nair & Ngouajio (2012) studied the effect of CC on soil microflora through CLPP analysis and found that both rye and rye-vetch mixture can affect the functional diversity of soil microbial community, enhancing soil biological activity. In our study, CC2 treatments produced the greatest increase in C sources consumption, probably due to the higher quantity and/or quality of root exudates provided by this CC mixture, compared with CC1. This effect was more evident at CC desiccation than at cash crop harvest, with a higher consumption of amino acids such as arginine, asparagine and lysine in CC2 treatment than in CC1 and the control. This suggests an increase in catabolic diversity when a legume species such as vetch is included in the mixture, probably linked to a major provision of N in the soil, which may be associated with an enhance of microbial growth (Garland *et al*., 2012). A further analysis of AUC using BD oxygen biosensor system (BDOBS-CLPP) would be interesting to fully understand functional diversity through C sources consumption as explained by Allegrini *et al*. (2015).

PCA based on C utilization profiles suggests that CC inclusion altered the functional diversity of the microbial community and increased the diversity of soil function. CC might provide the degradable C and energy sources required to increase rhizosphere microbial metabolic capacities and microbial diversity. Accordingly, Lagomarsino *et al*. (2012) found through CLPP analysis higher catabolic diversity in soils with greater quantity and quality of organic matter. Our results agree with those of Tian *et al*. (2013), who found that soils under CC inclusion showed a higher metabolic potential than traditional fallow system in cucumber crop. The ability of microorganisms to use a range of C substrates is fundamental to all the ecological functions in soils (Campbell *et al*., 2008). Therefore, our results evidence the positive effect of CC on the diversity of consumption of C sources, which permits characterization of soil microbial communities through their metabolic properties.

Microbial respiration and soil enzyme activities

Soil respiration is considered a bioindicator of soil quality and its assessment provides a measure of the overall potential of microbial activity (Dutta *et al*., 2010). In our study, microbial respiration showed a clear trend in response to the inclusion of CC, with control treatments exhibiting the lowest values at both sampling periods, as expected. Living roots and their exudates affect microbial activity, providing the energy sources needed to maintain a high microbial biomass and stimulating soil organic matter mineralization (Shahzad *et al*., 2015). Therefore, this rhizosphere effect and the extra organic matter provided by CC may have enhanced soil functionality in plots under CC mixtures, thus increasing soil microbial respiration. However, no differences were recorded between the two mixtures of species used as CC. Xi *et al*. (2012) studied the response of soil microbial respiration of tidal wetlands in China, and found that under certain conditions, in which the input of organic C was limited, greater input led to higher microbial respiration.

Soil enzyme activities reflect the cumulative effects of substrate availability, microbial activity and environmental conditions (Allison *et al*., 2011). In this regard, Chu *et al*. (2016) demonstrated that enzymes activities, such as DHA, are suitable indicators of soil quality due to their sensitivity to environmental factors. In this work, FDA hydrolysis and DHA were higher in soils under CC management in comparison with control treatments, which suggests an increased soil microbial activity generated by the diversification of the system. According to this, Reddy *et al*. (2003) reported that after three years with crimson clover or cereal rye as CC, soil had greater FDA than the soil without a CC. In their study, the crimson clover had a greater stimulatory effect on soil biology than cereal rye. Shahzad *et al*. (2015) found that roots exudates are used by microbes mostly to synthesize and release extra-cellular enzymes, thus mineralizing soil organic matter. Accordingly, the year-round presence of vegetation and the extensive root system of CC may increase the rhizosphere effect, stimulating microbial enzyme production. The higher amount of DHA observed in our research suggests an increase in soil microbial populations actively intervening in the degradation of organic matter, since DHA is a valid measure of the presence of viable

microorganisms and their oxidative capability (Rao *et al*., 2014). Our results are also consistent with those of Balota *et al*. (2014), who found that CC inclusion associated with no-tillage increased soil enzyme activities, particularly when a leguminous is used as CC. This general increase in enzyme activity produced by the inclusion of CC reflects an improvement in the functionality of microbial communities. The variations observed among CC treatments are due to the different species used as covering, since the type of CC can modify the development of the enzyme produced by microbial populations, promoting the growth of some microorganisms and inhibiting others (Elfstrand *et al*., 2007). In our study, there was a clear trend in soil enzyme activity regarding both CC mixtures, since oat, radish and vetch mix showed the highest enzyme activity values. It was evident that the inclusion of a legume species in the consociation of CC may have an improving effect on soil functionality. According to this, Velmourougane *et al*. (2013) assessed the impact of different cropping systems on DHA in different soil profiles and they found that legume crops showed the highest values of this enzyme activity. Also, Hamido & Kpomblekou (2009) found a greater enzyme activity in soils when tomato was preceded by crimson clover (*Trifolium incarnatum* L.), whereas black oat (*Avena strigosa* [Schreb.]) used as cover completely depressed the enzyme activity. Our results suggest that the use of three different plant species as CC increases soil microbial activity, revealing the benefit of a more diversified mixture on soil biological processes.

The seasonal enzymatic activity patterns recorded at cash crop harvest and CC desiccation may be associated with CC treatments as well as with temperature and moisture variations. Steinweg *et al*. (2012) demonstrated that both moisture and temperature can strongly control enzyme activity. They found that temperature was the dominant control when soil moisture was not limiting. Nevertheless, the effects of abiotic factors on soil biological proprieties are beyond the scope of our study.

Soil organic carbon

SOC stocks have been identified as good indicators of C dynamics under different management practices (Farage *et al*., 2007). Our results suggest that the use of CC generates an increase in SOC content, which brings several benefits to soil function in agrosystems by providing the energy resources needed for crop development. This effect was evident at both cash crop harvest and CC desiccation. Several studies have reported that management practices, such as crop rotation and the intensification of crop sequences by the use of CC mixtures, increase SOC sequestration into the soil (López-Fando & Pardo, 2011). Our analysis is consistent with the results obtained by Nascente *et al*. (2013), who demonstrated that the use of CC, such as millet, increased C concentrations in a cover crop-rice-cover crop-rice rotation in Brazil. Higashi *et al*. (2014) also reported that the use of winter rye (*Secale cereale*) showed statistically higher SOC content than hairy vetch (*Vicia villosa* Roth) used as CC in comparison with fallow. These differences in SOC in response to different CC species reported in the literature are probably due to the different quantity and quality of shoot and root biomass incorporated into the soil. However, unexpectedly, in our research, no differences were observed between the two CC mixtures regarding SOC, probably because vetch has a low C/N ratio and, therefore, may not have had a significant influence on SOC pool.

Recent studies have reported that microorganisms increase their respiration rate with an increase in C concentration in soils without nutrient limitations (Spohn & Chodak, 2015). This was also observed in our research, probably due to a higher amount of energy sources provided by CC in comparison with control treatments. Considering soil enzyme activities in our study, FDA and DHA were higher in soils under CC management than in the control treatment. This improvement was probably due to the increase in C input generated by CC, since microbes synthesize extracellular enzymes that decompose organic matter in order to obtain C (Hargreaves & Hofmockel, 2014). This may explain the positive correlation found between SOC and soil enzyme activities in this study. Our results are consistent with those of Nayak *et al*. (2007), who found a higher quantity of FDA and DHA in field plots enriched with C through compost inputs. Also Balota *et al*. (2014) concluded that a significant correlation between microbial variables and SOC is probably because higher SOC content supports larger microbial biomass, thus providing greater activity. In our study, these correlations were represented by a PCA, which also shows a negative correlation between BD and microbial activity variables, suggesting that the increase in microbiological activity enhanced by CC inclusion decreases soil BD. A decrease in BD values may indicate an increase in soil quality due to its relationships with other properties such as porosity, soil moisture and hydraulic conductivity (Dam *et al*., 2005). Our investigation suggests a positive effect of CC on physical fertility, since this agricultural technique increased AS, which is of great importance in protecting soil against erosion. According to this, Restovich *et al*. (2011) studied the impact of

different species of CC on soil physical variables finding a greater stability of the porous system in response to the inclusion of CC.

Our research evidenced that diversification of extensive agricultural systems through CC inclusion increases SOC pools, enhancing soil microbial activity and catabolic diversity, and, therefore, soil biological processes. Unlike other organic amendments, most of the C input from CC is added as roots, which contribute more effectively to the relatively stable carbon pool than above ground C-input (Kätterer *et al*., 2011). In this study, the increasing effect of SOC produced by CC was observed not only at CC desiccation, but also at cash crop harvest, which suggests that CC inclusion has an important role in cash crop nutrient supply. The observed higher increase in microbial activity and catabolic diversity in CC2 treatment than in CC1 supports that the inclusion of a legume species in the CC mixture produces important changes in soil functionality. The exudates released by roots are diverse and complex, and they can vary among plant species and its physiological conditions (Kumar *et al*., 2006). Therefore, the impact of different CC mixtures on SOC and microbial activity are probably due to the varying patterns of root exudation among plant species. This research evidences that the employment of a diverse mixture of CC including three different plant species is highly recommended to obtain an increase microbial activity and soil functionality in agrosystems where soybean crop prevails.

Grain yield

Even though it is accepted that CC has the potential to improve soil quality, contradictory information exists about the effect on yield of subsequent crops. The inclusion of CC in a cropping sequence was not found to reduce soybean yield under unlimited water availability for crop production (Ruffo *et al*., 2004; Restovich *et al*., 2012). However, Singer & Kohler (2005) reported reductions in soybean yield and shoot biomass accumulation with the use of rye (*Secale cereale* L.) as CC in Iowa (USA). Nevertheless, preliminary results indicate that, in the Pampas, the negative impact of CC on stored water would be negligible and would not produce significant decreases in crop yield (Fernandez *et al*., 2010). Although yield losses could occur in years with low amount of precipitation, the net benefits that CC provide to the system under normal conditions (no hydric stress) justify CC use in regions without hydric limitations.

In summary, the analysis of microbial functionality through different indicators was found to be a suitable tool to detect changes produced by the inclusion of CC. Our research showed that soil microbial respiration and enzyme activities were positively correlated with SOC content and are, therefore, sensitive variables to evaluate the impact of agricultural management changes. The CLPP method was also adequate to reveal the effects of management on the catabolic diversity of microbial communities in the analyzed soils. This study suggests that the inclusion of a leguminous species such as vetch in the CC mixture increases soil biological processes and promotes catabolic diversity. Since the inclusion of a CC mixture of three different species had no negative effects on cash crop grain yield, the use of this diversified mixture is highly recommended to enhance soil functionality (catabolic diversity, microbial respiration and soil enzyme activities) in regions without hydric limitations. In addition, CC inclusion in agricultural systems of the rolling Pampas has an improving effect on soil microbiological processes and soil physical properties, which can be recorded not only at CC desiccation but also as a residual effect at the end of cash crop cycle. Therefore, the use of CC is a promising agricultural technique to optimize soil quality in extensive agricultural systems.

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