Danilo S. Almeida[1*], Daniel Menezes-Blackburn[2,3], Benjamin L. Turner[4], Catherine Wearing[2], Philip M. Haygarth[2], Ciro A. Rosolem[1]

Urochloa ruziziensis cover crop increases the cycling of soil inositol phosphates

[1]*São Paulo State University, College of Agricultural Sciences, Department of Crop Science, Botucatu, 18610-307, Brazil*

[2]*Lancaster University, Lancaster Environment Centre, Lancaster, LA1 4YQ, UK*

[3]*Sultan Qaboos University, Department of Soils, College of Agricultural and Marine Sciences, Water and Agricultural Engineering, PO Box 34, Al-khod 123, Sultanate of Oman*

[4]*Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancon, Republic of Panama*

***Corresponding Author**

E-mail: daniloalmeidaagronomia@gmail.com

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Abstract

Ruzigrass (*Urochloa ruziziensis*) is a cover crop that is commonly used in Brazil and exudes high concentrations of organic acids from its roots, and is therefore expected to mobilize soil organic P such as inositol phosphates. However, is it not known if this can occur only under P deficient conditions. Specifically, we aimed to test the hypothesis that the degradation of inositol phosphates is increased by growing ruzigrass at two different P levels. To investigate this, we studied soil organic P in a nine-year old field experiment, with treatments consisting of ruzigrass or fallow during the soybean (*Glycine max*) off-season, with or without P addition. Organic P was extracted in NaOH-EDTA, followed by colorimetric quantification of organic P hydrolysable by phytase, and *myo*-inositol hexakisphosphate by hypobromite oxidation and HPLC separation. Ruzigrass dry matter yield increased by about 80% with P application. Ruzigrass reduced the concentration of phytase-labile P and *myo*-inositol hexakisphosphate, but only in soil receiving P. A corresponding increase in unidentified inositol phosphates, presumably representing lower-order esters, was also observed after ruzigrass in soil with P application. We deduce that the degradation of inositol phosphates under ruzigrass with P application is due to greater ruzigrass productivity in the more fertile treatment, increasing the release of root exudates that solubilize inositol phosphates and promote their decomposition by phytase. We conclude that ruzigrass cover cropping can promote the cycling of recalcitrant soil organic P, but only when fertility is raised to a sufficient level to ensure a productive crop.

Keywords: *myo*-inositol hexakisphosphate, organic phosphorus, no-till, cover-crops, hypobromite oxidation.

Introduction

The use of ruzigrass [*Urochloa ruziziensis* (R. Germ. and C.M. Evrard) Morrone and Zuloaga] in the off-season of cash crops in systems under no-till has been largely adopted in Brazil as a conservationist practice able to keep the soil covered over the off-season with minimal disturbance, to reduce soil erosion, promote weed control (Franzluebbers et al. 2014), and even to promote soil phosphorus (P) cycling, due to its high production of biomass (Almeida and Rosolem 2016). Recent studies have promoted the notion that growing certain species able to scavenge recalcitrant P forms can affect P cycling (Calegari et al. 2013; Merlin et al. 2014). Inorganic P fertilizers are the main source of P in agriculture, with a low use efficiency, and are obtained from phosphate rocks, which is a non-renewable source (MacDonald et al. 2011). Thus, the adoption of management practices able to improve P cycling is considered a step into sustainability of agricultural systems (Menezes-Blackburn et al. 2017). However, most species and microorganisms express their traits to scavenge recalcitrant P in P deficient soils (Li et al. 1997; Olander and Vitousek 2000), which is an unusual condition in agricultural soils. The high P application rates associated with the high P fixing capacity of tropical soils leads to most of added fertilizer P being fixed and accumulated in recalcitrant forms, such as inositol phosphates (MacDonald et al. 2011; Rodrigues et al. 2016). To seek and identify species able to promote soil P cycling, not only in P deficient soils is, therefore of potential benefit to sustainable management of agricultural areas in the tropics (Lagos et al. 2016; Menezes-Blackburn et al. 2017).

Ruzigrass has a C4-type photosynthesis and is well-adapted to low P availability due to a high P uptake efficiency (Rao et al. 1996). According to Onthong and Osaki (2006), the increase in the root:shoot ratio is the main P uptake mechanism in ruzigrass. Furthermore, ruzigrass can mobilize and take up soil recalcitrant P bound to Fe and Al (Merlin et al. 2015). The P uptake from non-labile P forms by ruzigrass may be related to an extensive root system

and the ability of roots to exude low-molecular weight organic acid anions, such as citrate and oxalate (Louw-Gaume et al. 2017; Wenzl et al. 2001). The high exudation of organic acids from ruzigrass roots may also mobilize organic P (P_o) forms (Martin et al. 2004). At least two mechanisms have been related to P mobilization by organic acid anions: the competition of organic acid for P adsorption sites on the surface of Fe and Al oxides, leading to P desorption, and the complexation of metal cations by organic acid anions inducing the solubilization of precipitated P (Hinsinger 2001; Jones 1998). The exudation of phosphatases from ruzigrass roots is also a mechanism to hydrolyze P_o, accounting for the high adaptation of ruzigrass to low P soils (Louw-Gaume et al. 2017).

Cycling of P_o has been considered as key factor to meet plant P demand, mainly in weathered soils under no-till, where the proportions of P_o tends to increase in the long-term (Rodrigues et al. 2016). In Brazilian Oxisols under no-till, it has been shown that P_o corresponds to 20 to 50% of total P (Costa et al. 2014; Olibone and Rosolem 2010; Rodrigues et al. 2016). This P_o portion consists in a range of compounds including orthophosphate esters, phosphonates, and phosphoric acid anhydrides (Condron et al. 2005). The orthophosphate monoesters include the inositol phosphates, which have been shown to be the dominant forms of P_o in many soils (Shears and Turner 2007; Turner et al. 2002), including Oxisols (Chapuis-Lardy et al. 2001). In particular, *myo*-inositol hexakisphosphate (also known as IP_6, $InsP_6$ or phytate) is one of the nine stereoisomers of inositol phosphates, with all the six hydroxyl groups esterified as phosphates moieties (Shears and Turner 2007). Among all soil P_o compounds, *myo*-inositol hexakisphosphate has the highest affinity to soil particles and the lowest bioavailability (Martin et al. 2004; Shang et al. 1996), which results in its accumulation in soils (Giaveno et al. 2010). The high soil-stability of *myo*-inositol hexakisphosphate is due to the capacity of numerous of its six phosphate groups to bind with cations and soil adsorption sites simultaneously (Celi et al. 1999; Shang et al. 1996). Inositol phosphate sorbs strongly to Fe

and Al oxides, kaolinite, montmorillonite, and soil organic matter (SOM) (Celi and Barberis 2007; Karathanasis and Shumaker 2009).

The extent to which inositol phosphates accumulate in soil depends on the presence of active phytate degrading enzymes (phytases), environmental conditions (pH, temperature, soil texture, and soil mineralogy), enzyme inactivation/inhibition, phytase mobility, and phytate solubility (Menezes-Blackburn et al. 2013; Nannipieri et al. 2011; Turner et al. 2002). Phytases are a special group of phosphatases that can initiate the dephosphorylation of *myo*-inositol hexakisphosphate (Shears and Turner 2007). In cases where the extracellular activity of phytase is present in soils, the phytase labile P ($P_{phy\text{-}lab}$) may be depleted and made available to plants (Yadav and Tarafdar 2004). A very small proportion of phytases (less than 1% of total phosphatase activity) was observed in ruzigrass roots by Louw-Gaume et al. (2010), and in a study conducted by Onthong and Osaki (2006) no phytase secretion by ruzigrass roots was observed. However, some phytase activity in soil under ruzigrass has been shown (Louw-Gaume et al. 2017), due to soil microbial activity, which provides an important mechanism by which plants can acquire P from recalcitrant P_o forms (Harrison 1987; Richardson et al. 2001). A higher phyatase activity has been observed mainly in the rhizosphere due to the favorable conditions for microbial proliferation (Yadav and Tarafdar 2004), which should also be favored in no-till, with higher SOM content(Alvear et al. 2005) .

Understanding the complex interrelations that affect soil P bioavailability is a challenge, but also a great opportunity for the development of technologies to reduce the accumulation of recalcitrant P_o forms in soil (Haygarth et al. 2018; Menezes-Blackburn et al. 2017). Most information on the soil P_o composition has come from ^{31}P nuclear magnetic resonance (^{31}P-NMR) spectroscopy of alkali-extractable P (Cade-Menun 2017). The use of high performance liquid chromatography (HPLC) to identify and quantify soil P_o forms has been proposed as an alternative technique, potentially more sensitive and less expensive than

^{31}P-NMR spectroscopy. Espinosa et al. (1999) previously used HPLC to identify and quantify P_o forms in soil leachates, but this method does not achieve high resolution and is unsuitable for the NaOH-EDTA extracts commonly used for ^{31}P-NMR spectroscopy. An alternative to improve the resolution and detection of soil P_o forms is the use of hypobromite oxidation of soil NaOH-EDTA extracts (Turner and Richardson 2004). The hypobromite oxidation digests non-phytate organic matter (Irving and Cosgrove 1981) and therefore eliminates organic matter interferences in HPLC separation of inositol phosphates, which may turn possible the use of a technique much more accessible than ^{31}P-NMR spectroscopy.

The objective of this study was to assess the effect of long-term soybean-ruzigrass rotation and P fertilizer application on the dynamics of soil P_o forms, mainly the *myo*-inositol hexakisphosphate, using an innovative approach by performing hypobromite oxidation of soil extracts and separation of *myo*-inositol hexakisphosphate with HPLC. Specifically, we aimed to test the hypothesis that the degradation of inositol phosphates is increased by growing a known soil P mobilizer cover crop (Ruzigrass) at two different P levels, in comparison to fallow. This study aims to improve the understanding of the soil P cycling and dynamics using crop rotation in the tropics.

Material and methods

Experimental site and treatments

A long-term field experiment established in 2006 was monitored for two years (2014 and 2015). The experiment is located in Botucatu, State of São Paulo, Brazil (22°50′00″ S; 48°25′31″ W; and altitude of 806 m) in an area that had been under no-till since 1998. The climate of the region, according to Köppen's classification, is Cwa (mesothermic with dry winter climate). The dry season is well defined from May to September. The average annual precipitation is 1,450 mm, with mean temperatures of 24 ºC during the warmest month and 13

151 ºC during the coldest month (Cepagri 2015). The soil is a Rhodic Hapludox (Soil Survey Staff,
152 2014) with 67% of sand, 12% of silt, and 21% of clay. From 2006, soybean [*Glycine max* (L.)
153 Merrill] has been grown in rotation with ruzigrass [*Urochloa ruziziensis* (R. Germ. and C.M.
154 Evrard) Morrone and Zuloaga] or fallowed during the off-season, with or without the
155 application of 26 kg ha^{-1} of P as triple superphosphate (TSP) in the soybean seed furrow.
156 Therefore, for ruzigrass, there was only the residual effect of P application to soybean. The
157 experiment design was a 2 × 2 factorial in randomized complete block design, with four
158 replications. Plots were 8 m long × 6 m wide.

159 The off-season period starts after soybean harvest in April and goes to November in the
160 soybean sowing. Ruzigrass was planted shortly after soybean harvest and all the plots (with
161 ruzigrass and fallow treatments) were desiccated using glyphosate (1.44 kg ha^{-1} a.i.) in
162 November. Spontaneous vegetation grew in the fallow plots, with the predominance of
163 *Rhynchelytrum repens* (Willd.) C.E. Hubb., and *Cenchrus echinatus* L., *Gnaphalium spicatum*
164 Lam. However, weed growth was random and sparse, with insignificant dry matter production
165 by the end of the off-season period. Four subsamples of ruzigrass shoots were randomly taken
166 using a 0.5 m x 0.5 m frame and combined into one sample per plot, oven dried at 60 ºC for 72
167 h, and then weighed to determine dry matter yield at November 2014 and 2015.

168 Soil samples were taken from depths of 0–5, 5–10, 10–20 and 20–40 cm in November
169 2014 and 2015, before chemical desiccation with herbicide. Six soil subsamples were randomly
170 taken from each plot using a 50 mm diameter core sampler, and were combined into one
171 composite sample per depth per field replication. The soil samples were air-dried and passed
172 through a 2-mm sieve for chemical analysis. Briefly, soil pH was determined in a 0.01 M
173 calcium chloride ($CaCl_2$) suspension (1:2.5 soil/solution), potential acidity (H+Al) was
174 estimated by the Shoemaker-McLean-Pratt (SMP) pH buffer method (Shoemaker et al. 1961),
175 Ca, Mg, K, and P were extracted using pearl resin according to Raij et al. (1986), cation

exchange capacity (CEC) was calculated as the sum of (H+Al)+Ca+Mg+K, and soil organic matter (SOM) was determined by chromic acid wet oxidation method (Walkley and Black 1934). Selected chemical characteristics of the soil of the experimental area are presented in Table 1.

Soil phosphorus extraction

Soil P was extracted with 0.25 M sodium hydroxide and 0.05 M disodium ethylenediaminetetraacetic acid (NaOH-EDTA). The NaOH-EDTA solution is a widely used extractant for soil P_o analysis and has been suggested as standard method for soil P_o extraction (Cade-Menun and Liu 2014). One gram of air-dried soil was extracted with 10 mL of NaOH-EDTA on a reciprocal shaker for 16 h. The extracts were then filtered using Whatman N1 filter paper and analyzed for total P (P_t), inorganic P (P_i), and organic P (P_o). The P_i concentration was determined immediately after extraction. Total P was obtained by autoclave digestion for 1 h at 121 ºC and 124 kPa, with potassium persulfate and sulfuric acid. Total P and P_i were obtained with the molybdate reactive P, which was quantified using the malachite green colorimetry of the digested and undigested extracts respectively (Van Veldhoven and Mannaerts 1987). The difference between P_t and P_i corresponds to the molybdate unreactive P, here termed as P_o. Figure 1 contains a scheme of the soil P extraction and the subsequent determinations (P_i, P_o, P_t, phytase labile P before hypobromination, steps of hypobromine oxidation of NaOH-EDTA soil extract, phytase labile P after hypobromination, and P speciation).

Phytase labile phosphorus

Phytase labile P ($P_{Phy\text{-}lab}$) was determined using a commercially available *Aspergillus niger* phytase (Natuphos, EC 3.1.3.8; BASF SE, Ludwigshafen, Germany) with high activity towards

sodium phytate (Wyss et al. 1999). The phytase was added to a final activity in excess of 50 nKat mL^{-1}. Briefly, 100 µL of the same soil extracts obtained with NaOH-EDTA were combined with 100 µL of phytase (100 nKat mL^{-1}) diluted in a buffer (50 mM acetate-acetic acid, pH 5.5) and incubated at 37 ºC for 16 h. Phytase labile P was measured via malachite green colorimetry. Phytase labile P was inferred by the difference of P_i content measured after the incubation of samples with phytases, and samples incubated with denatured phytases (autoclaved for 1 h at 121 ºC and 124 kPa) were used as blanks.

Hypobromite oxidation

The hypobromite oxidation was performed according to Irving and Cosgrove (1981), following the modifications described by Turner et al. (2012), in the same soil extracts obtained after soil P extraction with NaOH-EDTA. Prior to hypobromite oxidation, the pH of the soil extract was increased to 13 (to produce hypobromine after add the bromine), by adding 2 g of solid NaOH to a digestion tube containing 10 mL of soil extract, and the digestion tubes were cooled using an ice-bath. Then, 1 mL of pure bromine was added to the soil extract. After 1 hour, the extract was boiled at 140 ºC for 5 min to hydrolyse the non inositol P. To release all the bromine from the extract, the pH was lowered by adding 10 mL of 10 M hydrochloric acid. After bromine volatilization, the pH was adjusted to near 8.5 by adding 6 mL of 10 M NaOH. The final volume was adjusted to 30 mL with ultra-pure Milli-Q (MQ) water (Millipore). The phosphates from the extract were precipitated with barium, by adding 15 mL of barium acetate (10% w/v), and 15 mL of ethanol (50% v/v). The suspension was then centrifuged at 4000×g for 10 minutes. The supernatant was discarded, and the precipitate was washed with 30 mL of 50% ethanol, centrifuged, and the supernatant was discarded. The barium-phosphate precipitate were resuspended in 20 mL of amberlite IR120 cation exchange resin (hydrogen form) and 20 mL of MQ water, and shaken for 16 hours. Then, the extracts containing the remaining P forms

(inositol phosphates) and orthophosphate were separated from the resin by syringe-filtering (0.2 µm) and the final volume of each sample was adjusted to 35 mL. The extracts were neutralized and subjected to P_i, P_t determination using the malachite green colorimetry of the digested and undigested extracts respectively (Van Veldhoven and Mannaerts 1987), and phytase labile P determination as described in the previous section. Due to practical issues related to the large number of samples and the lengthiness of this protocol, only the soil depth of 0–10 cm was assayed with hypobromite oxidation.

Phosphorus speciation

The extracts obtained from the hypobromite oxidation were separated using High Performance Liquid Chromatography (HPLC), to determine the component P species, as in Espinosa et al. (1999). The separation was performed on an Agilent 1100 Series HPLC system (Agilent Technologies, California, U.S.), with an Agilent IonoSpher 5 A column (length 250 mm, internal diameter 4.6 mm). The extracts were pH adjusted to 5.5 with 1 M HCl and diluted in MQ water (1:10), and 30 µL of the diluted extract was injected into the HPLC column. The ionic linear gradient increased from 0.11 to 0.75 M NaCl over the 160-minute run, with a constant concentration of 0.5 mM EDTA and 50 mM MES. The flow rate was maintained at 0.5 mL min^{-1} and the buffers were pH adjusted to 5.5. A fraction collector was used to collect 1 mL of eluted sample every 2 min in glass vials. The eluted sample was then used for the quantification of P_t, P_i and $P_{Phy\text{-}lab}$ as described in the soil P extraction and phytase labile P sections. To confirm elution time of the P component peaks, a mixture of 100 µM sodium phosphate and 100 µM sodium *myo*-inositol hexakisphosphate, Sigma–68388 (Sigma-Aldrich, Missouri, U.S.), were used as a standard.

Statistical analysis

Data were first examined for homogeneity of variance using Levene's tests. Then, the results were subjected to analysis of variance by soil depth, considering a 2 × 2 factorial in randomized complete block design, with four replications, using a general linear model (Proc GLM) in SAS software (version 9.4, SAS Inst., North Carolina, U.S.). When the F test was significant ($p < 0.05$), treatment means were compared by Student's t-test ($p < 0.05$).

Results

Ruzigrass yield was almost twice as high in soil with P application than without P, in both years (Fig. 2). However, the yield in 2014 was lower than 2015, due to a low rainfall during the 2014 off-season.

On average, 40% of the P_t extracted with NaOH-EDTA corresponded to P_o in the 0–20 cm of soil depth, in both years. Despite the cumulative increase of SOM by growing ruzigrass as cover crop compared to fallow (Table 2), P_o concentration was not affected by ruzigrass. The concentration of P_t extracted with NaOH-EDTA was also not affected by the long term ruzigrass cover crop rotation (Table 3). The P fertilizer application affected P_t and P_o up to the 10–20 cm soil depth (Table 3). At the 20–40 cm soil depth, there was no effect of the treatments, and P_t and P_o averaged 57 and 23 mg kg^{-1}, respectively, in 2014 as well as in 2015.

The $P_{phy\text{-}lab}$ concentration before the hypobromite oxidation of the NaOH-EDTA was lower in the uppermost soil layer with ruzigrass than after fallow in soil fertilized with P in 2014 (Table 3). In 2015, $P_{phy\text{-}lab}$ was lower after growing ruzigrass than after fallow regardless of P application, at the depth of 0–5 and 5–10 cm. The $P_{phy\text{-}lab}$ concentration was lower with P fertilizer application than without (Table 3). There was no effect at the 20–40 cm soil depth, where the average $P_{phy\text{-}lab}$ was 5 and 6 mg kg^{-1}, in 2014 and 2015, respectively.

The $P_{phy\text{-}lab}$ concentration after hypobromite oxidation of the NaOH-EDTA extracts was markedly higher than before hypobromite oxidation in soil receiving P fertilizers (Table 4). However, there was no effect of ruzigrass on $P_{phy\text{-}lab}$ concentration after hypobromite oxidation (Table 4).

The HPLC retention time of P_i and the *myo*-inositol hexakisphosphate was on average 30 min apart, and phytate was the last observed peak. The confirmation of the *myo*-inositol hexakisphosphate peak was obtained both by co-elution with a standard and by phytase digestion of the collected HPLC fractions. A clear resolution of the soil extracts digested with bromine were obtained using the chosen HPLC method, as shown in Figure 3, nevertheless, peaks were somewhat broad, and resolution could be improved in further studies by coupling the HPLC to an inductively coupled plasma (ICP) for the online detection of the eluted phosphorus forms. Other unidentified small peaks were observed and labeled here as unidentified inositol phosphates.

The P_i concentration in the brominated NaOH-EDTA extracts was not different between ruzigrass and fallow treatments (Table 5). The concentration of unidentified inositol phosphates increased after ruzigrass in soil with P application compared to fallow, in both years. Growing ruzigrass resulted in lower *myo*-inositol hexakisphosphate concentration in soil with P application than in soil without P application (Table 5). In contrast, the fallowed soil with P application showed a higher *myo*-inositol hexakisphosphate concentration than soil without P application.

Discussion

Previous studies have shown that ruzigrass grown as a cover crop promotes the accumulation of P_o in the upper soil layers in the long term (Almeida and Rosolem 2016; Merlin et al. 2014), despite an increase in the phosphatase activity in soil (Rosolem et al. 2014). Here we intended to have a better understanding of field dynamics of P_o species under ruzigrass and specifically explore the following questions: how the concentration of *myo*-inositol hexakisphosphate and $P_{phy\text{-}lab}$ is affected by the agricultural system and if it gives insights on the field inositol phosphate cycling. How strong is the effect of P fertilizer application on these dynamics? Are there possible management perspectives from these results?

Phytase labile phosphorus and hypobromite oxidation

Soil P_o accounted for 40% of the total P, which is in agreement with that observed by Chapuis-Lardy et al. (2001) in weathered soils from Brazil. However, there was no effect of ruzigrass on soil P_o compared with soils kept fallow during off-season, despite the higher SOM content after ruzigrass. The soil P_o concentration is not necessarily correlated to the SOM concentration; nonetheless, there is usually a good correlation of soil P_o and total P concentration (Appelhans et al. 2016), as observed in the present study, where soil P_o concentration was higher with P application. It is worth mentioning that the increase of soil P_o by applying inorganic P fertilizers is a long-term effect, conversely, only an effective increase in the P_i should be expected in the short-term by applying inorganic P fertilizers. According to George et al. (2007), the continuous application of inorganic phosphate increases both soil P_i and P_o concentrations. The P_o increases with inorganic P fertilizer applications is a result of a higher P uptake by plants and the consequential greater amount of plant residue deposition (shoot and roots) as well as through the synthesis of P_o by soil microorganisms (Stewart and Tiessen 1987).

Interestingly, the soil $P_{phy\text{-}lab}$ and HPLC quantified *myo*-inositol hexakisphosphate were lower after ruzigrass in soil with P fertilizer application in comparison with both fallow and unfertilized treatments, indicating a synergistic effect of P fertilizer application and ruzigrass in increasing phytate bioavailability and cycling in these soils. However, despite a lower *myo*-inositol hexakisphophate concentration after ruzigrass, the P_t has not changed, but it is important to keep in mind that this is not the P_t from soil, it is the P_t extracted with NaOH-EDTA, corresponding to only a fraction of the soil P_t. It is also important to note that the $P_{phy\text{-}lab}$ is not necessarily *myo*-inositol hexakisphosphate, since the $P_{phy\text{-}lab}$ method use phytases with broad substrate specificity, and therefore includes non-phytate orthophosphate monoesters (Menezes-Blackburn et al. 2013). The $P_{phy\text{-}lab}$ method quantifies P_o lability, whilst the HPLC quantifies *myo*-inositol hexakisphosphate concentration, and perhaps HPLC could be used to quantify other hexakisphosphate isomers, such as *scyllo*-inositol hexakisphosphate.

The hypobromite oxidation of NaOH-EDTA soil extracts increased the measured $P_{phy\text{-}lab}$ concentration mainly in the treatment with P application, which is the opposite of the observed before hypobromite oxidation. This indicates that a significant part of $P_{phy\text{-}lab}$ in the NaOH-EDTA was not available to the phytase used. Is important to note that although the phytase used is only one of many phytases that might be found in soil, it has a wide substrate specificity. According to Hayes et al. (2000), both substrate availability and enzyme presence/activity are determinant of the hydrolysis of inositol phosphates in soils. Interactions with surface-reactive particles and the entrapment of phytases within humic molecules extracted from soil may act inhibiting the phytase activity (Nannipieri et al. 2011). A strong adsorption of inositol phosphates has been demonstrated in many soil compounds, such as calcite, illite, montmorillonite, goethite, and Al hydroxides, which limits the action of the enzyme (Menezes-Blackburn et al. 2013). According to Bowman and Moir (1993), NaOH promotes the solubilization of P_o, whereas EDTA is able to complex cations that binds P_o to

soil solid phase, overcoming a possible resistance of P_o to the extraction by NaOH. Possibly, part of the phytase added to the soil extract was inhibited by interactions with colloids remaining in the extracts after filtering and/or part of inositol phosphates precipitated or adsorbed to the soil colloids remaining in the extracts were not solubilized with NaOH-EDTA extraction, and were only accessed by the enzyme after hypobromite oxidation.

The concentration of P_i measured after hypobromite oxidation was about 16 mg kg^{-1} higher than before hypobromite oxidation. This increase in the P_i corresponds to 37% of the total P_o extracted with NaOH-EDTA, and this increase is due to the hydrolysis of P_o forms that are not resistant to the hypobromite oxidation (Irving and Cosgrove 1981). The hypobromite oxidation has been already successfully used to digest non-phytate organic matter in soil extracts for ^{31}P-NMR analysis (Turner et al. 2012; Turner and Richardson 2004). This degradation of other organic compounds by the hypobromite oxidation was crucial to ensure a pure extract, free of SOM interferents, and thereby allowing the use of HPLC for the determination of inositol phosphates in the soil NaOH-EDTA extracts.

New insights into the dynamics of myo-Inositol hexakisphosphate in soil with ruzigrass

Clearly, the HPLC analysis of NaOH-EDTA extracts after hypobromite oxidation allowed for new insights into the dynamics of recalcitrant P_o forms in soil. The higher concentration of $P_{phy\text{-}lab}$ observed after hypobromite oxidation was not necessarily exclusively due to *myo*-inositol hexakisphosphate, since the presence of other forms of P with retention times greater than orthophosphate and smaller than *myo*-inositol hexakisphosphate were found. These P forms were termed unidentified inositol phosphates, and are possibly products of *myo*-inositol hexakisphosphate degradation (e.g. *myo*-inositol 1,2,3,4,5-pentakisphosphate). The inositol phosphates (*myo*-inositol hexakisphosphate + unidentified inositol phosphates) accounted for

about 30% of the total P extracted with NaOH-EDTA, which is consistent with results from Cerrado soils in Brazil (22-39%) (Chapuis-Lardy et al. 2001).

The use of ruzigrass as a cover crop combined with P fertilizer applications resulted in lower soil concentrations of *myo*-inositol hexakisphosphate and higher concentrations of other forms of inositol phosphates, when compared to soil without P application. Several studies have shown that the application of soluble phosphates results in the suppression of phosphatase activity, with consequent increase of $P_{phy\text{-}lab}$ in the soil (George et al. 2007; Olander and Vitousek 2000; Rosolem et al. 2014), since the higher availability of P reduces the demand for P_o mineralization (Turner et al. 2002). However, in the present study, P was applied at soybean planting, about one year before soil sampling, thereby, this may be considered as a residual effect of P application. In addition, the concentration of P in fertilized soil is still considered low, resin-P is below 40 mg dm^{-3}. The low resin-P concentration is associated with the low total soil P concentration, on average 400 mg kg^{-1} (Almeida and Rosolem 2016), which is much lower than that observed in some European soils that receive large amounts of phosphate fertilizers (over 1000 mg kg^{-1}) over a long period (Menezes-Blackburn et al. 2017).

Ruzigrass may have favored the enzyme-producing microbial community capable of degrading inositol phosphates, since the various organic compounds exuded by ruzigrass roots, such as organic acid anions (Wenzl et al. 2001), are a source of energy for soil microorganisms (Hinsinger 2001; Menezes-Blackburn et al. 2016). Furthermore, the exudation of organic acid anions, such as citrate, by roots can complex cations and compete with soil sorption sites, preventing the adsorption of enzymes on soil particles (Hayes et al. 2000). Nevertheless, several factors affects the activity of enzymes in soil, such as electrostatic interactions, enzyme entrapment/adsorption, presence of inhibitors, and type of mineral precipitates formed with the substrate as revised by Nannipieri et al. (2011). A higher activity of phosphatases, including phytases, has been shown from soil microbial activity in the rhizosphere of ruzigrass (Louw-

Gaume et al. 2017; Rosolem et al. 2014). Even with the addition of P fertilizer, the activity of acid phosphatase in soil with ruzigrass is higher than in soil under fallow, as observed previously by Rosolem et al. (2014) using soil from the same experimental area of the present study. It is well stablished that the phosphatase activity is high in soil with ruzigrass (Simon et al. 2017). Compared to fallow and other cover crops, such as sorghum (*Sorghum bicolor*), millet [*Pennisetum glaucum* (L.) R. Br.], stylosanthes (*Stylosanthes* spp / CV. BRS), forage turnip (*Raphanus sativus* L.), crambe (*Crambe abyssinica* Hochst), soil with ruzigrass showed the highest activity of acid phosphatase (Simon et al. 2017). The increase of acid phosphatase activity in soil with ruzigrass has been correlated with an increase of P_i concentration (Louw-Gaume et al. 2010), showing an effective P cycling.

The lower concentration of $P_{phy\text{-}lab}$ after ruzigrass than after fallow, may also have been a result of the phytase activity from the ruzigrass roots. According to Louw-Gaume et al. (2010), ruzigrass roots show some phytase activity, which is not affected by P applications. Ruzigrass is a highly adapted species to tropical soils with low P availability (Begum et al. 2006), with roots able to increase P acquisition by physiological (Merlin et al. 2015) and morphological adjustments (Louw-Gaume et al. 2010; Wenzl et al. 2001). In addition, the continuous input of a great amount of ruzigrass residues, as well as the higher SOM concentration after ruzigrass than fallow may result in higher soil moisture, and fewer oscillations in soil temperature (Awan 1964), favoring the soil microbial activity. According to Nannipieri et al. (2011), it is well stablished that phosphatase activity is correlated with the content of SOM, which is usually higher in no-till systems. However, the lower concentration of $P_{phy\text{-}lab}$ after ruzigrass was only observed in soil with P application, which may indicate that P application was able to promote a more pronounced priming effect of soil phytate under ruzigrass than in fallow. As observed by Lagos et al. (2016), Luo et al. (2017), and Margenot et al. (2017) the P application does not necessarily suppress the activity of microorganisms-

harboring phosphatases. Some studies have attributed the increase in phosphatase activity to the increase in SOM (Alvear et al. 2005), P_o (Redel et al. 2007), and microbial biomass (Costa et al. 2013), when P is applied.

Since the ruzigrass yield was almost twice higher in soil with P application, a greater amount of organic compounds exuded by ruzigrass roots should be expected due to the increased root biomass, favoring the proliferation of microbial community, complexing cations (Lienhard et al. 2012), and solubilizing inositol phosphates (Gerke 2015; Martin et al. 2004). Despite a greater organic acid anion exudation per length of root be expected under low P soils than under high P soils, a greater amount of organic acid anion is also expected with the increase of root length. Additionally, relieving nutrient limitation by applying P favors the increase of crop biomass production and result in greater residue additions to soil (Margenot et al. 2017), and soil C may stimulate microbial activity because C has been found to be more limiting than P in some P-fertilized soils, such as in Kenya (Bünemann et al. 2004). According to Gerke (2015), future research considering P acquisition from *myo*-inositol hexakisphosphate should emphasize the mobilization of *myo*-inositol hexakisphosphate from the soil solid phase by root exudates, mainly di- and tricarboxylic acids, which may increase the solubility of *myo*-inositol hexakisphosphate.

Although *myo*-inositol hexakisphosphate represented on average only 12% of the total P extracted with NaOH-EDTA, the concentration decreased by 50% under the ruzigrass treatment when compared with the fallow in presence of P fertilization. This comparison is important, since fallow during off-season is still widely used in large areas in Brazil (Simon et al. 2017). Therefore, the P fertilized soybean-ruzigrass crop rotation, as recommended for soybean cultivation, can be an important management practice to induce the cycling of *myo*-inositol hexakisphosphate, considered the most recalcitrant P_o form in the soil. This cycling of *myo*-inositol hexakisphosphate may be even more effective in soils receiving organic

amendments, such manures, which has high *myo*-inositol hexakisphosphate concentration (Gatiboni et al. 2005). More studies are needed to evaluate the community of microorganisms and phytase activity in soil relating the expression of genes that codifies this enzyme (Lagos et al. 2016; Luo et al. 2017; Margenot et al. 2017), as well as the factors that favor the solubilization of *myo*-inositol hexakisphosphate and the development of microorganisms harboring phytases, such as the exudation of organic acid anions by ruzigrass roots.

Conclusion

Long-term soybean-ruzigrass crop rotation increases SOM content. However, the increase of SOM has no correlation with P_o and $P_{phy\text{-}lab}$, including *myo*-inositol hexakisphosphate accumulation in the soil. The soil *myo*-inositol hexakisphosphate concentration is reduced by growing ruzigrass as a cover crop in the soybean off-season, compared with fallow in the presence of P fertilizer applications, accepting the hypothesis that the degradation of inositol phosphates is increased by growing ruzigrass in soil receiving P applications. This is the opposite of what is usually observed: an increase of *myo*-inositol hexakisphosphate concentration when P fertilizers are applied. The P fertilizer application results in a great increase of ruzigrass biomass, which likely may have caused a higher exudation of organic acids and a consequent higher mobilization of recalcitrant P forms. The concentration of unidentified inositol phosphates was higher after ruzigrass than fallow. These unidentified inositol phosphates may be products of degradation of *myo*-inositol hexakisphosphates, which is also an evidence of the effect of ruzigrass in stimulating the degradation of soil *myo*-inositol hexakisphosphates.

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