

1 **Danilo S. Almeida^{1*}, Daniel Menezes-Blackburn^{2,3}, Benjamin L. Turner⁴, Catherine**
2 **Wearing², Philip M. Haygarth², Ciro A. Rosolem¹**

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4 ***Urochloa ruziziensis* cover crop increases the cycling of soil inositol**
5 **phosphates**

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

9 ²*Lancaster University, Lancaster Environment Centre, Lancaster, LA1 4YQ, UK*

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

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

14

15 ***Corresponding Author**

16 E-mail: danioloalmeidaagronomia@gmail.com

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27

28 **Abstract**

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

47

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

50

51 **Introduction**

52 The use of ruzigrass [*Urochloa ruziziensis* (R. Germ. and C.M. Evrard) Morrone and Zuloaga]
53 in the off-season of cash crops in systems under no-till has been largely adopted in Brazil as a
54 conservationist practice able to keep the soil covered over the off-season with minimal
55 disturbance, to reduce soil erosion, promote weed control (Franzluebbers et al. 2014), and even
56 to promote soil phosphorus (P) cycling, due to its high production of biomass (Almeida and
57 Rosolem 2016). Recent studies have promoted the notion that growing certain species able to
58 scavenge recalcitrant P forms can affect P cycling (Calegari et al. 2013; Merlin et al. 2014).
59 Inorganic P fertilizers are the main source of P in agriculture, with a low use efficiency, and
60 are obtained from phosphate rocks, which is a non-renewable source (MacDonald et al. 2011).
61 Thus, the adoption of management practices able to improve P cycling is considered a step into
62 sustainability of agricultural systems (Menezes-Blackburn et al. 2017). However, most species
63 and microorganisms express their traits to scavenge recalcitrant P in P deficient soils (Li et al.
64 1997; Olander and Vitousek 2000), which is an unusual condition in agricultural soils. The
65 high P application rates associated with the high P fixing capacity of tropical soils leads to most
66 of added fertilizer P being fixed and accumulated in recalcitrant forms, such as inositol
67 phosphates (MacDonald et al. 2011; Rodrigues et al. 2016). To seek and identify species able
68 to promote soil P cycling, not only in P deficient soils is, therefore of potential benefit to
69 sustainable management of agricultural areas in the tropics (Lagos et al. 2016; Menezes-
70 Blackburn et al. 2017).

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

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

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

101 and Al oxides, kaolinite, montmorillonite, and soil organic matter (SOM) (Celi and Barberis
102 2007; Karathanasis and Shumaker 2009).

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

119 Understanding the complex interrelations that affect soil P bioavailability is a
120 challenge, but also a great opportunity for the development of technologies to reduce the
121 accumulation of recalcitrant P_o forms in soil (Haygarth et al. 2018; Menezes-Blackburn et al.
122 2017). Most information on the soil P_o composition has come from ^{31}P nuclear magnetic
123 resonance (^{31}P -NMR) spectroscopy of alkali-extractable P (Cade-Menun 2017). The use of
124 high performance liquid chromatography (HPLC) to identify and quantify soil P_o forms has
125 been proposed as an alternative technique, potentially more sensitive and less expensive than

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

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

142

143 **Material and methods**

144 *Experimental site and treatments*

145 A long-term field experiment established in 2006 was monitored for two years (2014 and
146 2015). The experiment is located in Botucatu, State of São Paulo, Brazil (22°50'00" S;
147 48°25'31" W; and altitude of 806 m) in an area that had been under no-till since 1998. The
148 climate of the region, according to Köppen's classification, is Cwa (mesothermic with dry
149 winter climate). The dry season is well defined from May to September. The average annual
150 precipitation is 1,450 mm, with mean temperatures of 24 °C during the warmest month and 13

151 °C 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 °C 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 (CaCl₂) 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

176 exchange capacity (CEC) was calculated as the sum of (H+Al)+Ca+Mg+K, and soil organic
177 matter (SOM) was determined by chromic acid wet oxidation method (Walkley and Black
178 1934). Selected chemical characteristics of the soil of the experimental area are presented in
179 Table 1.

180

181 *Soil phosphorus extraction*

182 Soil P was extracted with 0.25 M sodium hydroxide and 0.05 M disodium
183 ethylenediaminetetraacetic acid (NaOH-EDTA). The NaOH-EDTA solution is a widely used
184 extractant for soil P_o analysis and has been suggested as standard method for soil P_o extraction
185 (Cade-Menun and Liu 2014). One gram of air-dried soil was extracted with 10 mL of NaOH-
186 EDTA on a reciprocal shaker for 16 h. The extracts were then filtered using Whatman N1 filter
187 paper and analyzed for total P (P_t), inorganic P (P_i), and organic P (P_o). The P_i concentration
188 was determined immediately after extraction. Total P was obtained by autoclave digestion for
189 1 h at 121 °C and 124 kPa, with potassium persulfate and sulfuric acid. Total P and P_i were
190 obtained with the molybdate reactive P, which was quantified using the malachite green
191 colorimetry of the digested and undigested extracts respectively (Van Veldhoven and
192 Mannaerts 1987). The difference between P_t and P_i corresponds to the molybdate unreactive P,
193 here termed as P_o. Figure 1 contains a scheme of the soil P extraction and the subsequent
194 determinations (P_i, P_o, P_t, phytase labile P before hypobromination, steps of hypobromine
195 oxidation of NaOH-EDTA soil extract, phytase labile P after hypobromination, and P
196 speciation).

197

198 *Phytase labile phosphorus*

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

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

208

209 *Hypobromite oxidation*

210 The hypobromite oxidation was performed according to Irving and Cosgrove (1981), following
211 the modifications described by Turner et al. (2012), in the same soil extracts obtained after soil
212 P extraction with NaOH-EDTA. Prior to hypobromite oxidation, the pH of the soil extract was
213 increased to 13 (to produce hypobromine after add the bromine), by adding 2 g of solid NaOH
214 to a digestion tube containing 10 mL of soil extract, and the digestion tubes were cooled using
215 an ice-bath. Then, 1 mL of pure bromine was added to the soil extract. After 1 hour, the extract
216 was boiled at 140 °C for 5 min to hydrolyse the non inositol P. To release all the bromine from
217 the extract, the pH was lowered by adding 10 mL of 10 M hydrochloric acid. After bromine
218 volatilization, the pH was adjusted to near 8.5 by adding 6 mL of 10 M NaOH. The final volume
219 was adjusted to 30 mL with ultra-pure Milli-Q (MQ) water (Millipore). The phosphates from
220 the extract were precipitated with barium, by adding 15 mL of barium acetate (10% w/v), and
221 15 mL of ethanol (50% v/v). The suspension was then centrifuged at 4000×g for 10 minutes.
222 The supernatant was discarded, and the precipitate was washed with 30 mL of 50% ethanol,
223 centrifuged, and the supernatant was discarded. The barium-phosphate precipitate were
224 resuspended in 20 mL of amberlite IR120 cation exchange resin (hydrogen form) and 20 mL
225 of MQ water, and shaken for 16 hours. Then, the extracts containing the remaining P forms

226 (inositol phosphates) and orthophosphate were separated from the resin by syringe-filtering
227 (0.2 μm) and the final volume of each sample was adjusted to 35 mL. The extracts were
228 neutralized and subjected to P_i , P_t determination using the malachite green colorimetry of the
229 digested and undigested extracts respectively (Van Veldhoven and Mannaerts 1987), and
230 phytase labile P determination as described in the previous section. Due to practical issues
231 related to the large number of samples and the lengthiness of this protocol, only the soil depth
232 of 0–10 cm was assayed with hypobromite oxidation.

233

234 *Phosphorus speciation*

235 The extracts obtained from the hypobromite oxidation were separated using High Performance
236 Liquid Chromatography (HPLC), to determine the component P species, as in Espinosa et al.
237 (1999). The separation was performed on an Agilent 1100 Series HPLC system (Agilent
238 Technologies, California, U.S.), with an Agilent IonoSpher 5 A column (length 250 mm,
239 internal diameter 4.6 mm). The extracts were pH adjusted to 5.5 with 1 M HCl and diluted in
240 MQ water (1:10), and 30 μL of the diluted extract was injected into the HPLC column. The
241 ionic linear gradient increased from 0.11 to 0.75 M NaCl over the 160-minute run, with a
242 constant concentration of 0.5 mM EDTA and 50 mM MES. The flow rate was maintained at
243 0.5 mL min^{-1} and the buffers were pH adjusted to 5.5. A fraction collector was used to collect
244 1 mL of eluted sample every 2 min in glass vials. The eluted sample was then used for the
245 quantification of P_t , P_i and $P_{\text{Phy-lab}}$ as described in the soil P extraction and phytase labile P
246 sections. To confirm elution time of the P component peaks, a mixture of 100 μM sodium
247 phosphate and 100 μM sodium *myo*-inositol hexakisphosphate, Sigma-68388 (Sigma-Aldrich,
248 Missouri, U.S.), were used as a standard.

249

250 *Statistical analysis*

251 Data were first examined for homogeneity of variance using Levene's tests. Then, the results
252 were subjected to analysis of variance by soil depth, considering a 2×2 factorial in randomized
253 complete block design, with four replications, using a general linear model (Proc GLM) in SAS
254 software (version 9.4, SAS Inst., North Carolina, U.S.). When the F test was significant ($p <$
255 0.05), treatment means were compared by Student's t-test ($p < 0.05$).

256

257 **Results**

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

261 On average, 40% of the P_t extracted with NaOH-EDTA corresponded to P_o in the 0–20
262 cm of soil depth, in both years. Despite the cumulative increase of SOM by growing ruzigrass
263 as cover crop compared to fallow (Table 2), P_o concentration was not affected by ruzigrass.
264 The concentration of P_t extracted with NaOH-EDTA was also not affected by the long term
265 ruzigrass cover crop rotation (Table 3). The P fertilizer application affected P_t and P_o up to the
266 10–20 cm soil depth (Table 3). At the 20–40 cm soil depth, there was no effect of the
267 treatments, and P_t and P_o averaged 57 and 23 mg kg⁻¹, respectively, in 2014 as well as in 2015.

268 The $P_{\text{phy-lab}}$ concentration before the hypobromite oxidation of the NaOH-EDTA was
269 lower in the uppermost soil layer with ruzigrass than after fallow in soil fertilized with P in
270 2014 (Table 3). In 2015, $P_{\text{phy-lab}}$ was lower after growing ruzigrass than after fallow regardless
271 of P application, at the depth of 0–5 and 5–10 cm. The $P_{\text{phy-lab}}$ concentration was lower with P
272 fertilizer application than without (Table 3). There was no effect at the 20–40 cm soil depth,
273 where the average $P_{\text{phy-lab}}$ was 5 and 6 mg kg⁻¹, in 2014 and 2015, respectively.

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

278 The HPLC retention time of P_i and the *myo*-inositol hexakisphosphate was on average
279 30 min apart, and phytate was the last observed peak. The confirmation of the *myo*-inositol
280 hexakisphosphate peak was obtained both by co-elution with a standard and by phytase
281 digestion of the collected HPLC fractions. A clear resolution of the soil extracts digested with
282 bromine were obtained using the chosen HPLC method, as shown in Figure 3, nevertheless,
283 peaks were somewhat broad, and resolution could be improved in further studies by coupling
284 the HPLC to an inductively coupled plasma (ICP) for the online detection of the eluted
285 phosphorus forms. Other unidentified small peaks were observed and labeled here as
286 unidentified inositol phosphates.

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

294

295 **Discussion**

296 Previous studies have shown that ruzigrass grown as a cover crop promotes the accumulation
297 of P_o in the upper soil layers in the long term (Almeida and Rosolem 2016; Merlin et al. 2014),
298 despite an increase in the phosphatase activity in soil (Rosolem et al. 2014). Here we intended
299 to have a better understanding of field dynamics of P_o species under ruzigrass and specifically
300 explore the following questions: how the concentration of *myo*-inositol hexakisphosphate and
301 $P_{phy-lab}$ is affected by the agricultural system and if it gives insights on the field inositol
302 phosphate cycling. How strong is the effect of P fertilizer application on these dynamics? Are
303 there possible management perspectives from these results?

304

305 *Phytase labile phosphorus and hypobromite oxidation*

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

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

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

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

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

359

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

361 Clearly, the HPLC analysis of NaOH-EDTA extracts after hypobromite oxidation allowed for
362 new insights into the dynamics of recalcitrant P_o forms in soil. The higher concentration of
363 $P_{\text{phy-lab}}$ observed after hypobromite oxidation was not necessarily exclusively due to *myo*-
364 inositol hexakisphosphate, since the presence of other forms of P with retention times greater
365 than orthophosphate and smaller than *myo*-inositol hexakisphosphate were found. These P
366 forms were termed unidentified inositol phosphates, and are possibly products of *myo*-inositol
367 hexakisphosphate degradation (e.g. *myo*-inositol 1,2,3,4,5-pentakisphosphate). The inositol
368 phosphates (*myo*-inositol hexakisphosphate + unidentified inositol phosphates) accounted for

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

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

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

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

404 The lower concentration of $P_{\text{phy-lab}}$ after ruzigrass than after fallow, may also have been
405 a result of the phytase activity from the ruzigrass roots. According to Louw-Gaume et al.
406 (2010), ruzigrass roots show some phytase activity, which is not affected by P applications.
407 Ruzigrass is a highly adapted species to tropical soils with low P availability (Begum et al.
408 2006), with roots able to increase P acquisition by physiological (Merlin et al. 2015) and
409 morphological adjustments (Louw-Gaume et al. 2010; Wenzl et al. 2001). In addition, the
410 continuous input of a great amount of ruzigrass residues, as well as the higher SOM
411 concentration after ruzigrass than fallow may result in higher soil moisture, and fewer
412 oscillations in soil temperature (Awan 1964), favoring the soil microbial activity. According
413 to Nannipieri et al. (2011), it is well established that phosphatase activity is correlated with the
414 content of SOM, which is usually higher in no-till systems. However, the lower concentration
415 of $P_{\text{phy-lab}}$ after ruzigrass was only observed in soil with P application, which may indicate that
416 P application was able to promote a more pronounced priming effect of soil phytate under
417 ruzigrass than in fallow. As observed by Lagos et al. (2016), Luo et al. (2017), and Margenot
418 et al. (2017) the P application does not necessarily suppress the activity of microorganisms-

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

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

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

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

450

451 **Conclusion**

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