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1 Effects of the earthworm *Pontoscolex corethrurus* on rice P nutrition and plant-available soil
2 P in a tropical Ferralsol

3

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19 **Abstract**

20 We conducted a greenhouse experiment in mesocosms for 28 d to assess the effects of the
21 endogeic earthworm *Pontoscolex corethrurus* on plant-available soil phosphorus (P) and rice
22 (*Oryza sativa*) P nutrition in a Malagasy Ferralsol. To assess plant-available soil P, we
23 determined the *L-value* by measuring the specific activity of P taken up from the soil by rice
24 and by applying the isotopic dilution principle. Despite earthworm mortality, *P. corethrurus*
25 significantly increased rice shoot biomass (+26%) and P nutrition (+65%), confirming that the
26 soil used for the experiment was P-deficient. The *L-value* also markedly increased from $6.8 \pm$
27 0.9 to 14.2 ± 1.3 mg P mesocosm⁻¹ in the presence of *P. corethrurus*. We estimated that the
28 orthophosphate ions released due to earthworm mortality contributed to 30% of the *L-value*
29 increase. We attributed the remaining 70% increase to the solubilization of native soil P
30 during its transit through the digestive tract. Thereafter, we discussed the sources of
31 uncertainty associated with the *L-value* calculation and their utilization to assess earthworm
32 effects on P availability in further studies.

33

34 Keywords: endogeic earthworms, ³²P-labeling, isotopic dilution, *L-value*, upland rice,
35 Madagascar

36

37 **1. Introduction**

38 Earthworms are involved in key soil functions (Lavelle et al., 2007; Liu et al., 2019). They
39 enhance soil nutrient availability for plants (Chapuis-Lardy et al., 2011; van Groenigen et al.,
40 2019). These terrestrial invertebrates can ingest considerable amounts of soil, for example, a
41 community of earthworms in Lamto savannas was reported to have consumed 1250 t ha⁻¹ yr⁻¹
42 (Lavelle et al., 1992). Endogeic earthworms can daily ingest soil quantity several times their
43 own weight, in the range of 1-10 g of dry soil g⁻¹ fresh weight day⁻¹, and specifically for
44 *Pontoscolex corethrurus*, a value between 1.5 and 3.15 g g⁻¹ d⁻¹ has been reported (Barois et
45 al., 1999). This can result in high nitrogen (N) mineralization, approximately 30-50 kg N ha⁻¹
46 yr⁻¹ (Lavelle et al., 2004), and considerably contribute to annual plant N uptake (Blouin et al.,
47 2013).

48 Despite increasing evidence and understanding, little is known about the distinct processes
49 involved in the effects of earthworms on phosphorus (P) flows (Le Bayon and Milleret, 2009;
50 Ros et al., 2017). Earthworms may contribute to diffusible inorganic P production by
51 enhancing microbial activity and organic P mineralization (*e.g.*, phosphatase activity), and/or
52 by changing the dynamics of orthophosphate ions via desorption in stable bio-structures
53 produced by earthworms due to shifts in soil pH during the gut transit (Vos et al., 2019), or
54 possibly due to competitive adsorption of elevated dissolved organic matter (Ros et al., 2017).
55 The effects of earthworms on P availability have, however, rarely been explored in the rice-
56 Ferralsol system. There is a dearth of research on this aspect and this is a serious concern,
57 considering that P is the main nutrient limiting plant production in acidic soils in tropical
58 regions (Hou et al., 2020; Raminoarison et al., 2020).

59 The *L-value* technique was proposed by Larsen (1952) to assess the fraction of soil P that was
60 plant-available. Its principle relies on the homogeneous labeling of orthophosphate ions in the
61 solid phase of the soil, that can later dilute orthophosphate ions in solution. The tracking is

62 performed by adding and by thorough mixing of a solution of carrier-free ^{32}P -ions (or ^{33}P -
63 ions) to the soil. The plant is used to sample the specific activity, i.e., ratio of radioactive P to
64 unlabeled P, of the P taken up by an individual plant after a defined period of growth.
65 Assuming no isotopic discrimination between labeled and unlabeled orthophosphate ions, the
66 *L-value* is calculated by applying the isotopic dilution principle. The *L-value* represents the
67 amount of plant-available exchangeable P. Thus, it is possible to quantify the effect of a
68 particular treatment on the fate of P from a particular soil source to the plant. The *L-value*
69 technique has been extensively used to assess and compare the amount of plant-available P in
70 soils (Bühler et al., 2003; Frossard et al., 1994; Pypers et al., 2006; Russell et al., 1957) and
71 the ability of plants to access different pools of soil P (Hedley et al., 1982; Hinsinger and
72 Gilkes, 1997). However, it has not been used thus far to assess the effects of earthworm
73 presence on plant-available soil P.

74 We conducted a greenhouse experiment to assess the effects of the endogeic earthworm *P.*
75 *corethrurus* (Rhinodrilidae) inoculation on rice (*Oryza sativa*) nutrition and the *L-value* in a
76 native Ferralsol of the Malagasy Highlands. We hypothesized that the earthworm-induced
77 solubilization of soil P during transit through the digestive tract enhanced rice P nutrition and
78 the *L-value*.

79

80 **2. Materials and Methods**

81 *2.1. Soil sampling and preparation*

82 We sampled the first 0-10-cm layer of a Ferralsol under natural grassland from the
83 experimental station of the “Laboratoire des Radio-Isotopes” (18°46'55" S, 47°32'46" E,
84 1274 m asl, Madagascar) in October 2016. The soil had a sandy-clay texture, with pH 5.5,
85 total organic C content of 20.8 g dry kg⁻¹, C:N ratio of 16, total P content of 380 mg dry kg⁻¹,
86 and Olsen P content of 4.7 mg dry kg⁻¹. Using the procedure described by Randriamanantsoa

87 et al. (2013) for P-deficient soil, the concentration of orthophosphate ions in solution was
88 determined as 0.004 mg P L⁻¹.

89 The soil was air-dried to a constant weight (7 d), sieved to obtain 2 mm particle size, re-
90 humidified with sterile water to 50% of its water-holding capacity, and finally autoclaved for
91 10 min at 100°C to kill earthworms (adults, juveniles, and cocoons). The autoclaved soil was
92 air-dried to a constant weight (7 d) and sieved again at 2 mm. A microbial suspension was
93 prepared from fresh soil using a two-step protocol (see Ranoarisoa et al. (2018) for details on
94 soil preparation). Briefly, we provided 28.2 mg-N and 3.4 mg-P per kg dry soil as bacterial
95 biomass N and P, and 28.2 mg-N as NH₄Cl and 3.4 mg-P as KH₂PO₄ per kg dry soil as a
96 basal mineral fertilization.

97

98 2.2. Biological materials

99 We focused on the endogeic earthworm *P. corethrurus* (Ponton), which is highly abundant in
100 Madagascar, and the main species in cropped soils in the highlands of Madagascar
101 (Razafindrakoto et al., 2010). *P. corethrurus* belongs to the family Rhinodrilidae. It is a
102 peregrine species found throughout the tropics. It is 5-8 cm in length, weighing 0.7 g on
103 average at the adult stage. It is an endogeic geophagous species living in an exceptionally
104 substantial range of soil and climate environments and easily adapts to man-made
105 agrosystems. Individuals of *P. corethrurus* were collected using a spade from the
106 experimental station and inoculated in the same fresh soil (not autoclaved) for 5 d. Before
107 inoculation, earthworms were rinsed three times with distilled water. Twenty individuals were
108 weighed after 48 h at 65°C. Dried tissues were finely and manually ground and total P in the
109 *P. corethrurus* tissues was analyzed (3 replicates) after calcination at 550°C for 5 h and
110 digestion with nitric acid (65%). The P concentration was determined in the digest solutions
111 using the molybdenum blue method (Murphy and Riley, 1962).

112

113 2.3. Soil ³²P labeling

114 The soil was labeled with a carrier-free orthophosphate ³²P ion radionuclide source delivered
115 in HCl 1M (NEX054010MC (323 TBq mmol⁻¹), PerkinElmer®, Belgium), and was diluted in
116 water with an activity rate of approximately 0.1 mCi kg⁻¹ dry soil. The soil was mixed by
117 hand for 15 min in a large plastic container, and 500 g of ³²P-labelled dry soil was introduced
118 in 1-L plastic mesocosms. A basal nutrient solution containing N, Ca, K, Mg, S, Fe, Mn, Zn,
119 Cu, Co, B, and Mo was added to achieve 70%-80% of soil water holding capacity. Eight
120 seeds of rice (cultivar B22) were planted in each mesocosm and three rinsed adults of *P.*
121 *corethrurus* were inoculated per +Ponto mesocosm (treatment with *P. corethrurus*, see
122 below). Thereafter, 100 g of acid-washed sand was provided at the top of the soil to optimize
123 germination conditions and to protect workers from radiation.

124

125 2.4. Experimental design

126 We conducted a mesocosm experiment in a greenhouse with two treatments, namely a control
127 without earthworms (-Ponto) and a treatment containing *P. corethrurus* (+Ponto). To limit the
128 handling of radioactive materials, ten mesocosms (five without earthworms and five with
129 earthworms) without the addition of ³²P were used to perform analyses on fresh soil and plant
130 samples. Furthermore, ten supplementary mesocosms (five without earthworms and five with
131 earthworms) with the addition of ³²P were used to perform *L-value* analyses. All mesocosms
132 (labeled or not labeled) were treated in the same way. The mesocosms were randomly placed
133 in the greenhouse. During the period of rice culture, the soil moisture content was controlled
134 twice a day to maintain constant conditions. The climatic conditions (mean T (°C) and mean
135 PAR) within the greenhouse were monitored with a climatic station (WatchDog Series
136 Weather Stations, USA) (Supporting Information 1).

137

138 2.5. Measurements from unlabeled mesocosms

139 After 28 d, whole rice plants were removed, and the associated roots, seed residues, and
140 shoots of rice were separated, washed, dried at 65°C for 48 h, and weighed. Total P content in
141 shoots, roots, and seeds was determined after crushing, calcination at 550°C for 5 h, and
142 digestion of plant tissues with nitric acid (65%). P content in plant tissues was determined in
143 the digest solutions using the molybdenum blue colorimetric method (Murphy and Riley,
144 1962). We analyzed the total Mg, K, and Ca contents in the digest solutions using atomic
145 absorption spectroscopy (Thermo Scientific iCE 3000 SERIES, China). Total C and N
146 contents in plants (shoots and roots) were determined using a CHNS/O elemental
147 microanalyzer (Flash 2000 Series, CHNS/O 122 Analyzers Thermo Scientific, IRCOF,
148 France). We collected the earthworms (individuals and cocoons) by manually sieving the
149 fresh soil. We determined the survival rate of *P. corethrurus* by comparing the final
150 earthworm abundance to the initial number. Soil aggregation was determined by sieving air-
151 dried soil at 2 mm, which corresponded to a mass proportion of soil greater than 2 mm
152 (Blanchart, 1992). We used soil aggregation as a proxy for *P. corethrurus* activity.

153

154 2.6. Measurements from labeled mesocosms

155 After 28 d, only plant shoots were removed from the labeled mesocosms, dried at 65°C for 48
156 h, and weighed. The P content in shoots was determined as described above. The shoot
157 digests were analyzed for ³²P radioactivity using a liquid scintillation analyzer (Tri-Carb 2800
158 TR, PerkinElmer) with a liquid scintillation cocktail (Ultima Gold XR). The ³²P activities, R
159 (introduced radioactivity), and r (remaining radioactivity in harvested shoots), were corrected
160 for radioactive decay. The specific activity of shoot P was calculated from the ratio between
161 r/R and shoot P uptake. The *L-value* was calculated by applying the isotopic dilution

162 principle, that is, the specific activity of the *L-value* equalled the specific activity of P taken
163 up by a plant, as demonstrated in the follow equation:
164 $R/L = r / (P_{\text{shoot}} - P_{\text{seed}}) \Rightarrow L = (P_{\text{shoot}} - P_{\text{seed}}) / (r/R)$,
165 with r representing the ³²P-radioactivity in rice shoot at harvest, R representing the ³²P-
166 radioactivity of the initially applied to soils to each mesocosm, and P_{seed} representing the
167 seed-borne P in rice shoots. To correct the *L-value* for P_{seed}, we followed the procedure
168 proposed by Achat et al. (2014). Briefly, the fraction of P in rice shoots derived from seed P
169 mobilization (P_{seed}) was calculated by subtracting the initial amount of P in seed at seedling
170 and the remaining amount of P in seed residues found in the not-labeled soils (see above) after
171 28 d of cultivation. This amount was proportionally distributed between the shoot and root of
172 rice on a mass-basis. We did not handle fresh soil from the labeled mesocosms due to
173 radioactivity.

174

175 *2.7. Statistical analysis*

176 Mean and standard deviation (SD) values were computed per treatments for all variables. We
177 performed Student's *t*-tests with earthworm treatments as a factor for all variables using the R
178 software (R Core Team, 2016). Statistical significance was set at $P < 0.05$.

179

180 **3. Results and discussion**

181 *3.1. Earthworm survival rates and effect on soil aggregation*

182 Neither Ponto individuals nor cocoons, nor any decaying tissues were observed in unlabeled
183 mesocosms at the end of the experiment. The last observation of fresh casts was conducted on
184 the 19th day after the commencement of the experiment. We found casts in +Ponto soils and
185 the soil aggregation rate increased from 3.5% in -Ponto to 15.5% in +Ponto treatments (Fig. 1.
186 A). In this soil and for similar experimental conditions, Chapuis-Lardy et al. (2009) measured

187 1.5 g casts produced per day and per earthworm. Therefore, we determined the production of
188 casts during the 19-day period of Ponto activities as 85.5 g corresponding to 17.7% of the soil
189 weight. This result was in agreement with the percentage of aggregates measured.

190

191 *3.2. Effects of Pontoscolex corethrurus on rice biomass and nutrition*

192 Despite mortality, *P. corethrurus* significantly increased rice shoot biomass (+26%), total
193 biomass (+15%), and rice P nutrition (+65%) (Table 1). Owing to the application of the basal
194 nutrient solution, rice N, K, Ca, and Mg did not differ significantly across the -Ponto and
195 +Ponto treatments (Table 1). However, a general trend was observed with a slight increase in
196 N, K, Ca, and Mg uptake in the +Ponto treatment. These results were in agreement with those
197 of the previously reported studies (Hauser, 1993). These effects are likely due to the release
198 and decomposition of Ponto tissues at death, considering the content of N (1.8%), K (0.5%),
199 Ca (0.2%), and Mg (0.2%) in earthworm tissues is important for such studies (Lourdumary
200 and Uma, 2013).

201

202 *3.3. Effects of Pontoscolex corethrurus on L-values*

203 In our study, the P content in seed residuals at the end of the experiment was 7.9 (± 0.49) $\mu\text{g P}$
204 mesocosm^{-1} in -Ponto and 13.1 (± 0.41) $\mu\text{g P}$ mesocosm^{-1} in +Ponto (Table 1), which
205 represented an average of 2.6% of the initial amount of P in seeds at seedling. After
206 subtracting it from the P accumulation in rice shoots for 28 d of culture, *L-values* were
207 calculated considering only the P derived from plant-available soil P. The *L-values* were 6.8
208 mg P mesocosm^{-1} soil in -Ponto and 14.2 mg P mesocosm^{-1} in +Ponto, which represented an
209 increase of +109% when comparing the treatment to the control.

210 The decomposition of Ponto tissues after their death releases nutrients, especially

211 orthophosphate ions. This process is usually recognized as negligible even if earthworm

212 survival in pot experiments can be below 100% (Bertrand et al., 2015; Chassé et al., 2019;
213 Paul et al., 2012; Vos et al., 2019), and on a few occasions, it can be below 60% (Derouard et
214 al., 1997; Fonte et al., 2019; Huang et al., 2015; Ratsiatosika et al., 2019). In our study, we
215 considered the initial amount of P in +Ponto (2.21 mg P mesocosm⁻¹ soil), which was released
216 by the Ponto decomposition. This contributed to an increase in the *L-value* by ~30%
217 ($2.21 \times 100 / (14.2 - 6.8)$) with a maximal value of 38.1% and the lowest value of 22.1% (Fig. 1.
218 B). The remaining 70% of the *L-value* increase could be attributed to the Ponto activities,
219 which partially solubilized soil P through the transit in their digestive tract.

220 The following two questions arise from these observations: 1) What are the mechanisms that
221 might explain the increase in the *L-value*?; and 2) What are the possible sources of
222 uncertainty that could lead to overestimation or underestimation of the *L-value*?

223 We could not clearly identify the main mechanisms that could lead to solubilization of native
224 soil P during its transit in the digestive tract. Little is known regarding the distinct processes
225 involved in their effects on P flows (Le Bayon and Milleret, 2009; Ros et al., 2017). P in
226 acidic soils, including soils containing orthophosphate ions, forms complexes with Al- and
227 Fe-oxyhydroxides; additionally, P precipitates as amorphous and crystalline Al and Fe
228 minerals and organic P compounds (Sample et al., 1980; Turner, 2006). According to this
229 variable speciation, several biological and physicochemical processes might transform native
230 P soil and modify the equilibrium between added orthophosphate ³²P ions that dilute native
231 ³¹P soil. Since about 31% of the total P in Malagasy soils is available in the organic form
232 (Turner, 2006), *P. corethrurus* may contribute to the release of orthophosphate ions in
233 solution that diffuse at the solid-to-solution interface by enhancing mineralization of soil
234 organic matter (Chapuis-Lardy et al., 2011; Ghosh et al., 1999; Le Bayon and Milleret, 2009).
235 For instance, Coulis et al. (2014) observed lower amounts of soil organic P extracted with
236 NaOH, but higher water-soluble inorganic P content when earthworms (*Allobophora*

237 *chlorotica*) were present in a durum wheat-chickpea intercropping glasshouse experiment.

238 Additionally, dissolution of phosphate minerals in the native soil could occur in the digestive

239 tract. However, this effect is likely minor because the internal pH is close to 7.0 (Barois and

240 Lavelle, 1986). Transitions in the digestive tract may also affect soil-to-solution dynamics of

241 orthophosphate ions by changing soil properties in stable bio-structures produced by

242 earthworms due to shifts in soil pH (Vos et al., 2019) or due to competitive adsorption of

243 elevated dissolved organic matter (Ros et al., 2017).

244 The substantial increase (+109%) of the *L-value* associated with Ponto inoculation should be

245 considered with considerable caution even after correction for P_{seed} because several sources of

246 uncertainties can disturb its calculation and interpretation. In this study, especially, one source

247 of bias could be the result of non-homogeneous soil-casts- (*P. corethrurus* after

248 decomposition) mixture, since the mass of casts represented 85.5 g and the remaining soil in

249 mesocosms amounted to 414.5 g. Both *P. corethrurus* casts and soil fertilized by the addition

250 of *P. corethrurus* decaying tissues create small volumes favorable to growth of rice roots and

251 absorption of orthophosphate ions. As a result, the specific activity of P taken up by rice may

252 be preferentially absorbed from these small volumes having a lower specific activity than that

253 in other soil volumes. These local effects do not validate the basic hypothesis to calculate the

254 *L-value*, in which a homogeneous mixture is necessary. Consequently, it is likely that this

255 leads to a significant overestimation of the *L-value*. The same drawback was encountered in

256 studies on the capacity of different crop species to access poorly available soil P as assessed

257 by the *L-value* technique (Hocking, 2001). The *L-value* was considerably larger with white

258 lupin compared to *L-values* obtained with other crop species. This increase was explained by

259 the ability of white lupin which developed specialized proteoid roots in P-deficient soil, which

260 exuded, locally in their rhizosphere, considerable amounts of citrate or citric acid to mobilize

261 soil P. This indicated that P uptake was substantial in proteoid root clusters than that in apical

262 segments of non-proteoid roots (Neumann et al., 1999). Thus, although the isotopic tracing
263 technique has shown to be an extremely powerful and precise method in many soil-plant-
264 fertilizer studies, it should be used with caution in ecological studies investigating the role of
265 earthworms on P availability.

266 In summary, earthworms considerably increased plant growth and P nutrition. Considering all
267 interpretation precautions cited above for the *L-value*, we can conclude that most of the
268 earthworm-driven P availability is attributed to *P. corethrurus* activities that partially
269 solubilize soil P through soil transit in its digestive tract. However, our experiment showed
270 that the contribution of dead *P. corethrurus* decomposition to plant P nutrition was significant
271 (approximately 30%). The effects of earthworms on P availability are dependent on species
272 and ecological categories (Suárez et al., 2004). Thus, the relative contribution of the different
273 mechanisms quantified in this study should not be considered generic. However, according to
274 the worm density inoculated and the duration of the pot experiments, the survival of
275 earthworms should be considered while evaluating plant-available P.

276

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285

286

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Fig. 1. (A) Soil aggregation rate according to earthworm presence expressed in mass percentage. Whiskers correspond to standard deviation. (B) Contribution of the decomposition pathway on plant P nutrition following earthworm inoculation expressed as percentage of the *L-value*.

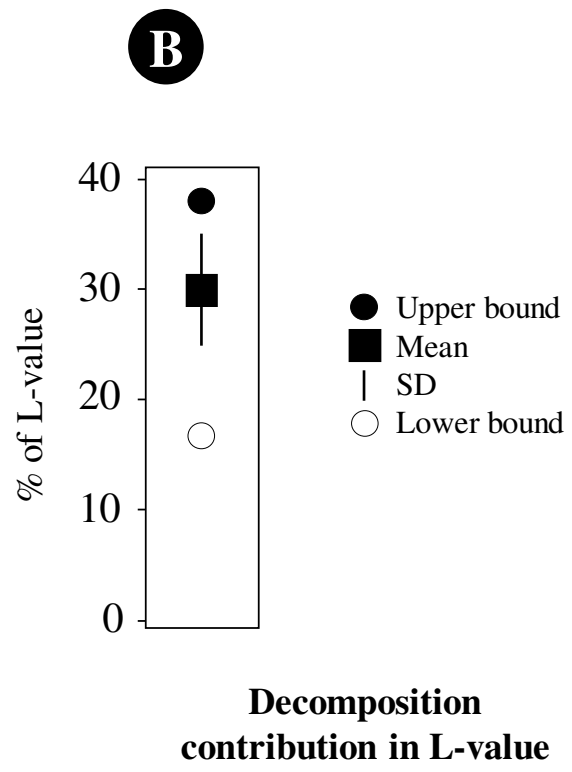
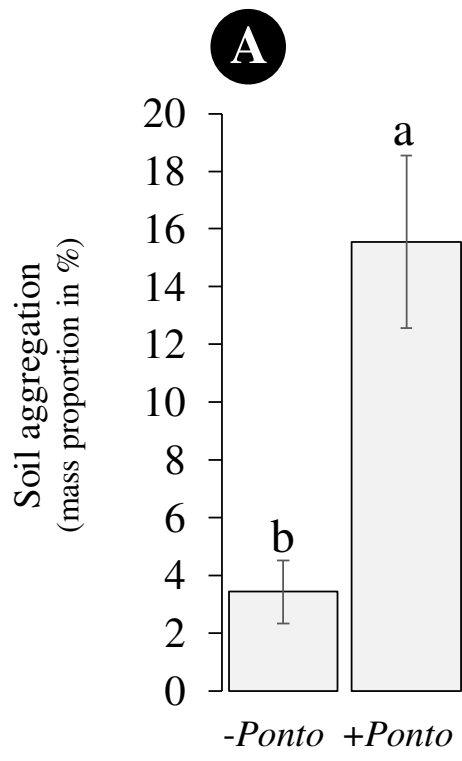


Table 1. Plant variables measured after 28 d of growth in the presence (+*Ponto*) or absence (-*Ponto*) of the earthworm *Pontoscolex corethrurus* in a Ferralsol from Madagascar.

Variables	Unit	Treatments					
		- <i>Ponto</i>		+ <i>Ponto</i>			
Shoot biomass	mg mesocosm ⁻¹	537	(65)	b 677	(36)	a	
Root biomass	mg mesocosm ⁻¹	441	(71)	a 445	(27)	a	
Shoot : root ratio	ratio	1.2	(0.2)	a 1.5	(0.1)	a	
Total biomass	mg mesocosm ⁻¹	978	(118)	b 1122	(29)	a	
Shoot P amount	mg mesocosm ⁻¹	0.37	(0.05)	b 0.61	(0.09)	a	
Root P amount	mg mesocosm ⁻¹	0.24	(0.05)	b 0.32	(0.02)	a	
Total P amount	mg mesocosm ⁻¹	0.61	(0.09)	b 0.95	(0.11)	a	
Shoot L-value	mg-P mesocosm ⁻¹	6.8	(0.9)	b 14.2	(1.3)	a	
Shoot N amount	mg mesocosm ⁻¹	15.1	(2.3)	a 16.1	(0.7)	a	
Shoot K amount	mg mesocosm ⁻¹	19.1	(2.6)	a 20.2	(1.1)	a	
Shoot Ca amount	mg mesocosm ⁻¹	1.8	(0.5)	a 1.9	(0.1)	a	
Shoot Mg amount	mg mesocosm ⁻¹	1.2	(0.2)	a 1.2	(0.1)	a	
Shoot N:P ratio	ratio	38.8	(6.7)	a 22.4	(2.7)	b	
Shoot Ca:Mg ratio	ratio	1.4	(0.1)	a 1.5	(0.1)	a	
Shoot K:(Ca+Mg) ratio	ratio	6.5	(1.16)	a 6.3	(0.6)	a	
Final P in seed residues	μg-P mesocosm ⁻¹	7.9	(0.49)	b 13.1	(0.41)	a	
Earthworm P content	mg-P dry g ⁻¹	5.3	(2.24)				
Initial P in seed at seedling stage	mg-P dry kg ⁻¹	1343	(73)				

Mean (SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Letters (a and b) indicate significant differences between '-*Ponto*' and '+*Ponto*' treatments according to the Student t test at $P < 0.05$.