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

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HAL Id: hal-03152409
https://hal.inrae.fr/hal-03152409

Submitted on 2 Jan 2023

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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 ±

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, ^{32}P-labeling, isotopic dilution, *L-value*, upland rice,

35 Madagascar

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2

1. Introduction

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

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

The *L-value* technique was proposed by Larsen (1952) to assess the fraction of soil P that was plant-available. Its principle relies on the homogeneous labeling of orthophosphate ions in the 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 NH_4Cl and 3.4 mg-P as KH_2PO_4 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* (Ponto), 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

2.3. Soil ^{32}P labeling

The soil was labeled with a carrier-free orthophosphate ^{32}P ion radionuclide source delivered

in HCl 1M (NEX054010MC (323 TBq mmol^{-1}), PerkinElmer®, Belgium), and was diluted in

water with an activity rate of approximately 0.1 mCi kg^{-1} dry soil. The soil was mixed by

hand for 15 min in a large plastic container, and 500 g of ^{32}P-labelled dry soil was introduced

in 1-L plastic mesocosms. A basal nutrient solution containing N, Ca, K, Mg, S, Fe, Mn, Zn,

Cu, Co, B, and Mo was added to achieve 70%-80% of soil water holding capacity. Eight

seeds of rice (cultivar B22) were planted in each mesocosm and three rinsed adults of *P.*

corethrurus were inoculated per +Ponto mesocosm (treatment with *P. corethrurus*, see

below). Thereafter, 100 g of acid-washed sand was provided at the top of the soil to optimize

germination conditions and to protect workers from radiation.

124

2.4. Experimental design

We conducted a mesocosm experiment in a greenhouse with two treatments, namely a control

without earthworms (-Ponto) and a treatment containing *P. corethrurus* (+Ponto). To limit the

handling of radioactive materials, ten mesocosms (five without earthworms and five with

earthworms) without the addition of ^{32}P were used to perform analyses on fresh soil and plant

samples. Furthermore, ten supplementary mesocosms (five without earthworms and five with

earthworms) with the addition of ^{32}P were used to perform *L-value* analyses. All mesocosms

(labeled or not labeled) were treated in the same way. The mesocosms were randomly placed

in the greenhouse. During the period of rice culture, the soil moisture content was controlled

twice a day to maintain constant conditions. The climatic conditions (mean T (°C) and mean

PAR) within the greenhouse were monitored with a climatic station (WatchDog Series

Weather Stations, USA) (Supporting Information 1).

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 ^{32}P radioactivity using a liquid scintillation analyzer (Tri-Carb 2800

158 TR, PerkinElmer) with a liquid scintillation cocktail (Ultima Gold XR). The ^{32}P 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    $R/L = r / (P_{shoot} - P_{seed}) => L = (P_{shoot} - P_{seed}) / (r/R)$,

165   with r representing the ^{32}P-radioactivity in rice shoot at harvest, R representing the ^{32}P-

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) µg P

204   mesocosm^{-1} in –Ponto and 13.1 (±0.41) µg 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-value*s were

207   calculated considering only the P derived from plant-available soil P. The *L-value*s 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   (2.21×100/(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 ^{32}P ions that dilute native

231   ^{31}P 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-value*s 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.

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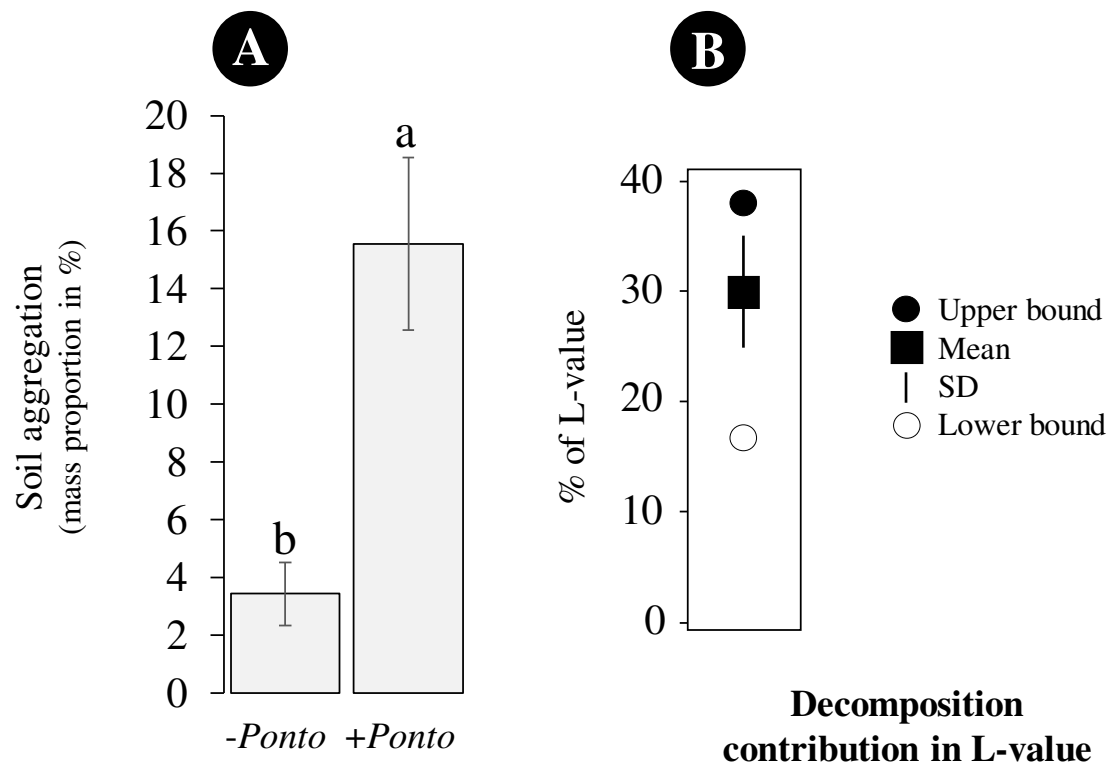
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Fig. 1. (A) Soil aggregation rate according to earthworm presence expressed in mass percentage. Whiskers correspond to standard deviation. (B) Contribution of the decomposition pathway on plant P nutrition following earthworm inoculation expressed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

A

Soil aggregation (mass proportion in %)

b

a

-*Ponto*   +*Ponto*

B

% of L-value

● Upper bound
■ Mean
| SD
○ Lower bound

**Decomposition
contribution in 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					
		-*Ponto*			+*Ponto*		
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	$mg\text{-}P\ mesocosm^{-1}$	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	$\mu g\text{-}P\ mesocosm^{-1}$	7.9	(0.49)	**b**	13.1	(0.41)	**a**
Earthworm P content	$mg\text{-}P\ dry\ g^{-1}$	5.3	(2.24)				
Initial P in seed at seedling stage	$mg\text{-}P\ dry\ kg^{-1}$	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 $P < 0.05$.