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

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Abstract

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

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

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

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

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

2. Materials and Methods

2.1. Soil sampling and preparation

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

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

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

2.2. Biological materials

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

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.

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).

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, IROF,
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

principle, that is, the specific activity of the *L-value* equalled the specific activity of P taken up by a plant, as demonstrated in the follow equation:

$$R/L = r / (P_{\text{shoot}} - P_{\text{seed}}) \Rightarrow L = (P_{\text{shoot}} - P_{\text{seed}}) / (r/R),$$

with *r* representing the ³²P-radioactivity in rice shoot at harvest, *R* representing the ³²P-radioactivity of the initially applied to soils to each mesocosm, and *P_{seed}* representing the seed-borne P in rice shoots. To correct the *L-value* for *P_{seed}*, we followed the procedure proposed by Achat et al. (2014). Briefly, the fraction of P in rice shoots derived from seed P mobilization (*P_{seed}*) was calculated by subtracting the initial amount of P in seed at seedling and the remaining amount of P in seed residues found in the not-labeled soils (see above) after 28 d of cultivation. This amount was proportionally distributed between the shoot and root of rice on a mass-basis. We did not handle fresh soil from the labeled mesocosms due to radioactivity.

2.7. Statistical analysis

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

3. Results and discussion

3.1. Earthworm survival rates and effect on soil aggregation

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

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

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

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

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

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

The decomposition of Ponto tissues after their death releases nutrients, especially orthophosphate ions. This process is usually recognized as negligible even if earthworm

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

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

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

chlorotica) were present in a durum wheat-chickpea intercropping glasshouse experiment. Additionally, dissolution of phosphate minerals in the native soil could occur in the digestive tract. However, this effect is likely minor because the internal pH is close to 7.0 (Barois and Lavelle, 1986). Transitions in the digestive tract may also affect soil-to-solution dynamics of orthophosphate ions by changing soil properties in stable bio-structures produced by earthworms due to shifts in soil pH (Vos et al., 2019) or due to competitive adsorption of elevated dissolved organic matter (Ros et al., 2017).

The substantial increase (+109%) of the *L-value* associated with Ponto inoculation should be considered with considerable caution even after correction for P_{seed} because several sources of uncertainties can disturb its calculation and interpretation. In this study, especially, one source of bias could be the result of non-homogeneous soil-casts- (*P. corethrurus* after decomposition) mixture, since the mass of casts represented 85.5 g and the remaining soil in mesocosms amounted to 414.5 g. Both *P. corethrurus* casts and soil fertilized by the addition of *P. corethrurus* decaying tissues create small volumes favorable to growth of rice roots and absorption of orthophosphate ions. As a result, the specific activity of P taken up by rice may be preferentially absorbed from these small volumes having a lower specific activity than that in other soil volumes. These local effects do not validate the basic hypothesis to calculate the *L-value*, in which a homogeneous mixture is necessary. Consequently, it is likely that this leads to a significant overestimation of the *L-value*. The same drawback was encountered in studies on the capacity of different crop species to access poorly available soil P as assessed by the *L-value* technique (Hocking, 2001). The *L-value* was considerably larger with white lupin compared to *L-values* obtained with other crop species. This increase was explained by the ability of white lupin which developed specialized proteoid roots in P-deficient soil, which exuded, locally in their rhizosphere, considerable amounts of citrate or citric acid to mobilize soil P. This indicated that P uptake was substantial in proteoid root clusters than that in apical

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

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

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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*.

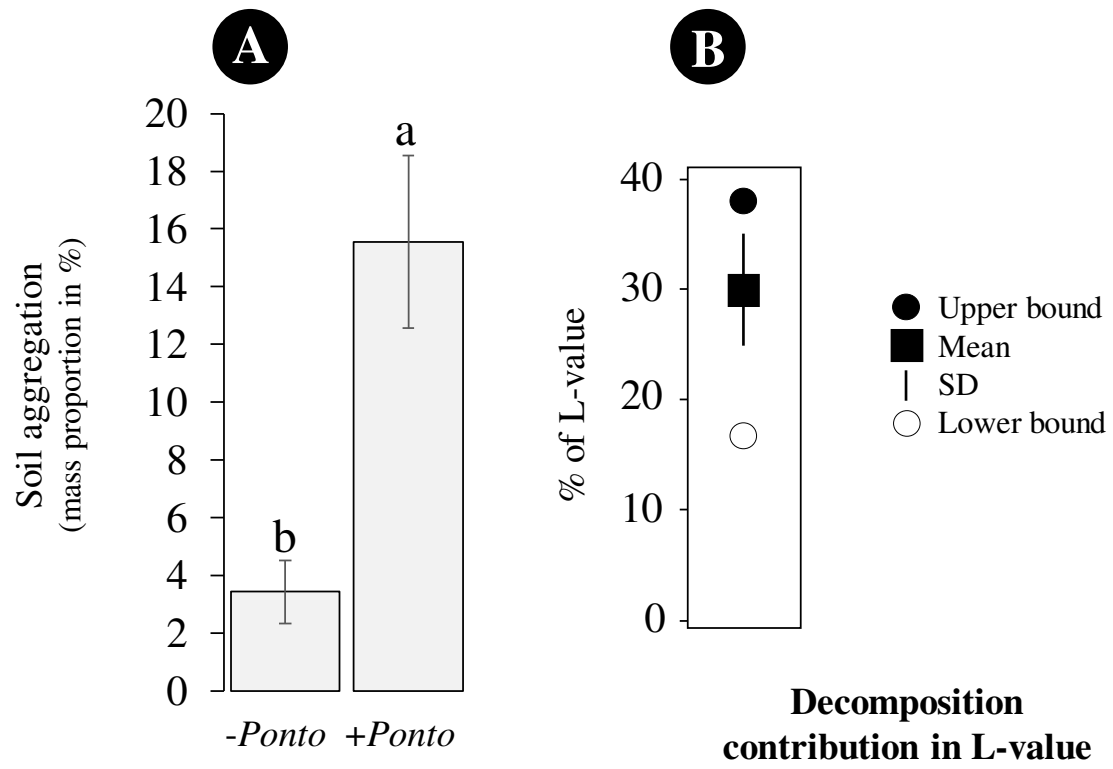


Table 1. Plant variables measured after 28 d of growth in the presence (+*Ponto*) or absence (-*Ponto*) of the earthworm *Pontoscolex corethrurus* in a Ferralsol from Madagascar.

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 $P < 0.05$.