

## Article

# Earthworm Inoculation Improves Upland Rice Crop Yield and Other Agrosystem Services in Madagascar

Onja Ratsiatosika <sup>1</sup>, Malalatiانا Razafindrakoto <sup>1</sup>, Tantely Razafimbelo <sup>1</sup>, Michel Rabenarivo <sup>1</sup>, Thierry Becquer <sup>2</sup>, Laetitia Bernard <sup>2</sup>, Jean Trap <sup>2</sup> and Eric Blanchart <sup>2,\*</sup> 

<sup>1</sup> Laboratoire des Radio-Isotopes, University of Antananarivo, BP 3383, Route d'Andraisoro, Antananarivo 101, Madagascar; onja.ratsiatosika@gmail.com (O.R.); malalasraz@yahoo.fr (M.R.); tantely.razafimbelo@gmail.com (T.R.); miarabenarivo@yahoo.fr (M.R.)

<sup>2</sup> Eco&Sols, Institut Agro, University Montpellier, CIRAD, INRAe, IRD, 2 Place Viala, 34060 Montpellier, France; thierry.becquer@ird.fr (T.B.); laetitia.bernard@ird.fr (L.B.); jean.trap@ird.fr (J.T.)

\* Correspondence: eric.blanchart@ird.fr

**Abstract:** The effects of earthworm inoculation and cropping systems on upland rice systems were examined over a four-year period in the Highlands of Madagascar. Each year, endogeic earthworms *Pontoscolex corethrus* (Rhinodrilidae) were inoculated (EW+) at a density of 75 ind m<sup>-2</sup> or were not inoculated (EW0). Inoculation was tested in three cropping systems: conservation agriculture (CA) and traditional tillage with or without residues restitution. Soil and plant properties were measured during the first three years while soil biological properties were assessed at the fourth year. At the end of the experiment, earthworm density was three-fold higher in EW+ than in EW0, demonstrating the success of the inoculation. Earthworm density was more important in CA than in tillage systems. Earthworm inoculation had higher significant effects on soil and plant properties than cropping systems. Earthworm inoculation had positive effects on soil macroaggregation (+43%), aboveground biomass (+27%), rice grain yield (+45%), and N grain amount (+43%). Intensifying earthworm activity in field conditions to meet the challenge of ecological transition is supported by our study.

**Keywords:** *Pontoscolex corethrus*; soil ecological intensification; Ferralsols; soil macroaggregates; rice growth; rice yield



**Citation:** Ratsiatosika, O.; Razafindrakoto, M.; Razafimbelo, T.; Rabenarivo, M.; Becquer, T.; Bernard, L.; Trap, J.; Blanchart, E. Earthworm Inoculation Improves Upland Rice Crop Yield and Other Agrosystem Services in Madagascar. *Agriculture* **2021**, *11*, 60. <https://doi.org/10.3390/agriculture11010060>

Received: 11 December 2020

Accepted: 11 January 2021

Published: 13 January 2021

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## 1. Introduction

Earthworms are involved in many ecological functions leading to the delivery of ecosystem services. Their intense feeding and moving activities have strong impacts (i) on biogeochemical cycles, i.e., carbon (C) transformations [1], nutrient cycling [2], and silicon (Si) availability [3]; (ii) on soil structure and physical properties through the production of biogenic structures [4,5]; (iii) on the control of pests and disease [6,7]; and (iv) on the activity of other soil biota, bacteria [8], fungi [9], nematodes [10], soil microarthropods [11], and (v) on plant growth [12].

Based on this knowledge, the consideration of earthworms for increasing the delivery of ecosystem services from agrosystems (what we call here 'agrosystem services') has been proposed for many years [13]. Following their potentially beneficial effects on functions and services, earthworms are generally perceived as animals to be sustained in agricultural systems. It is very likely that the manipulation of the soil community is key to successful restoration of terrestrial ecosystems [14,15]. The main approach consists of changing practices: reducing practices detrimental to earthworms (tillage, pesticides) and developing practices beneficial to earthworms (liming, organic fertilization) [16]. Another approach is inoculation, defined here as the deliberate introduction of living organisms in a given soil with the aim that this action will result in beneficial changes in the dynamics and equilibrium of the environment (i.e., biofertilization). Inoculation, as an active biostimulation

approach, has barely been developed at large scales and over a long period [17]. For earthworms, inoculation can concern either cocoons, juveniles, adults, or micro-environments transplantation [18]. Earthworm inoculation needs to be associated to inputs of organic matter aimed at restoring the habitat and favoring earthworm development [19].

Theoretically, it seems possible to inoculate earthworms in all types of soil, under different climates, and for different land-uses, provided that earthworms exist locally and can adapt to soil disturbance. Different trials have been realized in temperate and tropical regions (e.g., [20] in the UK; [21] in Peru; [22] in India; [23] in the West Indies). However, large-scale experiments focusing on agricultural fields are rare and, to our knowledge, only one such assay has been carried out in tea plantations in India [24]. In this experiment, the inoculated earthworms belong to the peregrine species *Pontoscolex corethrurus* (Rhinodrilidae), and earthworms were inoculated in trenches where tea prunings were buried. This technology was called bio-organic fertilization and resulted in a 240% increase in tea production. An initial objective was to inoculate earthworms at a biomass higher than 30 g m<sup>-2</sup> live weight to positively improve/restore soil and plant functions [25]. In the experiment in India, earthworms were reproduced in large covered beds with a cost of production estimated at 3.6 euros kg<sup>-1</sup> (i.e., 1400 euros to produce the equivalent of 400 kg live weight per hectare). At this cost, it appears that earthworm inoculation should only be applied to high value crops; this also explains why this technology did not spread worldwide.

In the Highlands of Madagascar, upland crops face many edaphic, environmental, climatic, and economic constraints. Ferralsols are very infertile with many limiting nutrients, especially P, phosphates being rapidly sorbed on clay minerals and oxides, but also N, Ca, Mg [26]. Cereal crop production on these soils is limited by the poor soil fertility, the strong development of pests and diseases (white grubs, blast disease, striga, etc.), and the very low financial ability of smallholder farmers to buy fertilizers and biocides. Fertility improvement is realized with cow manure and, more rarely, pig manure, and crop residues.

Earthworms from Madagascar are relatively well known, with intense surveys being carried out in recent years [27–30]. In the Highlands, the main species occurring in anthropized environments are exotic species such as *P. corethrurus* (Rhinodrilidae), *Dichogaster bolau*, *Dichogaster saliens* (Acanthodrilidae), and *Amyntas corticis* (Megascolecidae). Earthworm densities are higher in perennial than in annual crops, and higher in non-tilled than in tilled situations [31]. *P. corethrurus* is an endogeic peregrine species found all over the tropics; it is considered as invasive by some authors and tolerates a wide range of environmental conditions [32]. This species has been shown to deeply modify soil structure and soil C and N cycles [32,33], and to increase P availability [34]. This last function is especially important for P-fixing soils such as the Ferralsols from the Highlands of Madagascar. A recent experiment demonstrated that the activity of *P. corethrurus* can also promote plant health and can especially protect rice against blast disease in Madagascar [7]. A consequence of soil modifications by this species generally results in improved plant growth [21,35].

We hypothesized that soil habitat restoration associated with *P. corethrurus* inoculation could improve crop production and other agrosystem services, such as C sequestration, and erosion control through an improvement of soil macroaggregation. In the present study, we followed a four-year experimental upland rice (*Oryza sativa*) crop where earthworms were inoculated or not in three different agricultural systems. Earthworms were inoculated each year at the beginning of the cropping season. Cropping systems were: traditional tillage without residues, traditional tillage with residues, and conservation agriculture, i.e., without tillage and with rotation of an association of maize with the legume dolichos (*Dolichos purpureus* L.). We hypothesized that earthworm inoculation and consequences on agrosystem services would be more efficient in conservation systems and less efficient in tilled situations without residues.

## 2. Materials and Methods

### 2.1. Study Site

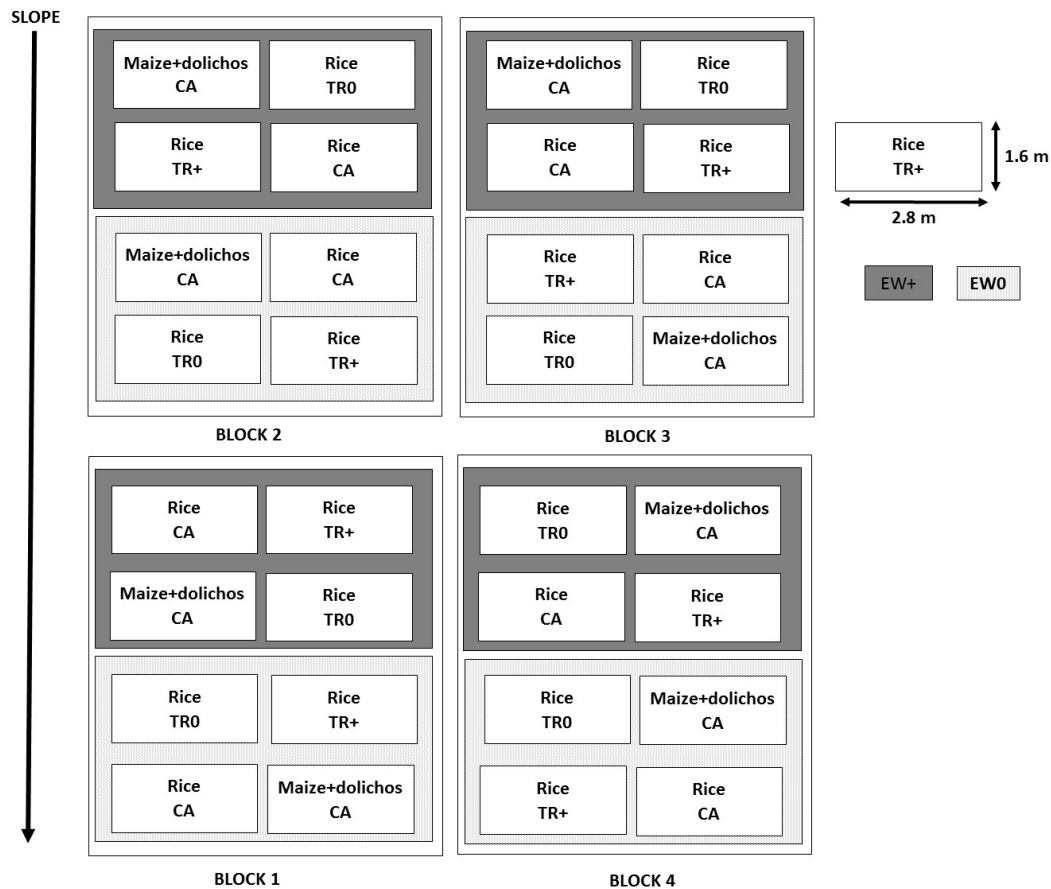
The experiment was conducted at Lazaina (18°46′55.59″ S, 47°32′46.3″ E, 1274 m above sea level), in the Analamanga region of the Malagasy highlands. The climate is a tropical altitude climate with an average annual temperature of 20 °C and mean annual rainfall of 1300 mm. The area is characterized with a warm and humid season from October to April and a cool and dry season from May to September. The upland rice cropping season starts at the end of November and ends in April. The study was conducted over four growing seasons: 2013–2014, 2014–2015, 2015–2016, and 2016–2017. Rainfall was higher in the period 2014–2015 (1232 mm during the cropping season, December–April) than in other periods (888–1082 mm).

Soils are Ferralsols with high contents of kaolinitic clays and Al- and Fe-oxides. Under natural vegetation, mainly composed of grasses *Aristida* sp., soil has a sandy-clay texture with 33% clay, a pH of 5.5, a total organic C content of 20.8 g kg<sup>-1</sup>, a total N content of 1.3 g kg<sup>-1</sup>, a C:N of 16, a total P content of 380 mg kg<sup>-1</sup>, an Olsen P content of 7.12 mg kg<sup>-1</sup>, and exchangeable K, Ca, and Mg contents of 30.7, 120.7, and 28.3 mg kg<sup>-1</sup>, respectively. The cationic exchange capacity (CEC) was 1.34 cmol<sup>+</sup> kg<sup>-1</sup> and the water-holding capacity (WHC) was 0.49 g g<sup>-1</sup> dry soil.

### 2.2. Experimental Design and Plot Preparation

The experimental plot was under *Aristida* savanna for more than 30 years before being cropped with maize between 2006 and 2012; this was followed by a one-year fallow period before our experiment started in 2013. Four plots of the previous experiment were thus retained to represent the four blocks of our experiment. On the first year of experiment, all subplots were tilled, with an ‘angady’ (traditional iron spade) at 20–25 cm depth and then carefully homogenized within the block, to homogenize the soil. Two factors were tested in this experiment: (i) the inoculation of earthworms with two modalities: with earthworm inoculation (EW+) or without earthworm inoculation (EW0), and (ii) the cropping system with three modalities: traditional tillage with rice residues restitution each year (TR+), traditional tillage without rice residues restitution (TR0), and a conservation agriculture system (CA) including two rotations: rice in rotation with an association of maize (*Zea mays*) + dolichos (*Dolichos purpureus*) and association of maize + dolichos in rotation with rice. The soil was not tilled in the CA systems after the first cropping year. The experiment is thus composed of 32 subplots (2 earthworm treatments, 4 cropping systems, 4 replicates, Figure 1). Each subplot had a 5.6 m<sup>2</sup> surface area. In the experimental field, the land presented a slope of 8%. To limit soil disturbance and soil runoff flows, all treatments with earthworms (EW+) in a block were gathered in the upper part of the block, while treatments without earthworms were gathered in the lower part. We are aware that this choice will have consequences on the statistics used; thus, this ‘plot position’ was considered in further statistical analysis. The area gathering the four treatments of EW+ were surrounded by a metal sheet buried vertically in the soil up to 40 cm deep and protruding 5 cm above the ground surface. The aim of this was to prevent earthworms from escaping EW+ treatments.

The sowing took place in mid-December. Rice holes were 20 cm apart with 7–8 seeds in each hole, equivalent to 80 kg seed ha<sup>-1</sup>. We used the rice cultivar ‘FOFIFA 161’. For maize + dolichos, rows of maize were alternated 45 cm with rows of dolichos. Maize seeds (‘IRAT 200’, 3 per hole) and dolichos seeds (*D. purpureus* with brown seeds, 3 per hole) were 45 cm apart on a row and 40 cm apart on a row, respectively. Rice and maize + dolichos were both fertilized with manure and NPK fertilizer (11% N, 22% P, 16% K) at doses equivalent to 10 Mg ha<sup>-1</sup> and 200 kg ha<sup>-1</sup>, respectively. At the end of the vegetative stage (stem elongation), urea was supplied at a rate of 50 kg ha<sup>-1</sup>. Weeding was carried out after one month of sowing and was repeated every 15 days until the flowering stages. The main insect pests (*Apoderus humeralis*, Coleoptera Curculionidae) were monitored and hand-removed.



**Figure 1.** Experimental design. Treatments are abbreviated as follows: EW0: without earthworm inoculation, EW+: with earthworm inoculation, TR0: traditional tillage without rice residues restitution, TR+: traditional tillage with rice residues restitution, CA: conservation agriculture.

### 2.3. Earthworm Inoculation

For this experiment, we used the species *P. corethrurus*, largely distributed in the anthropized areas of the Highlands. They were collected from an area close to our experiment (less than 100 m), under eucalyptus trees growing in the ‘Bozaka’ savanna. Earthworms were kept in a basin with wet soil. Each year, we introduced earthworms in subplots EW+ to reach a density of 75 ind m<sup>-2</sup>, slightly higher than the threshold (60 ind m<sup>-2</sup>, i.e., 30 g m<sup>-2</sup>) proposed by [25] to obtain a significant effect of earthworms on plant growth. This means a quantity of 6680 earthworms for the whole experiment, i.e., 350 per subplot, each year. Three days, with five people, were necessary to reach this number each year. After collecting the worms, they were transferred to buckets with water, in order to count them and to remove dead or injured worms and earthworms from other species.

### 2.4. Soil and Plant Analyses

Soil was carefully characterized at the beginning of the experiment in order to get an initial state of soil properties. Soil (0–10 cm) was removed using an auger, with 6 samples per subplot gathered to make a composite sample. Soil C (Walkley–Black method) and N (Kjeldhal method) contents, and exchangeable (resin) inorganic P content (Pi) were measured in the laboratory. Bulk density (0–10 cm) was also measured with 2 cylinders per subplot.

At the end of each cropping season, four samples per subplot were gathered to make a composite sample for analyses. Bulk density was also measured with 2 cylinders per subplot. Soil macroaggregation was also measured by dry sieving at 2 mm [36]. We

calculated the percentage of macroaggregates as the weight of aggregates retained by the sieve, divided by the whole weight of sieved soil.

Rice height was regularly measured, each year, at each important development stage, i.e., beginning (one month after sowing) and end (two months after sowing) of the tillering stages, end of flowering stage (3 months after sowing). At rice harvest (end of April), aboveground biomass (sum of shoot and grain biomasses) and grain yield were measured for all plants except dead plants and the border line. Shoots and grains were kept and air-dried for further N and P analyses. Root biomass was measured by hand-collecting all visible roots from two soil monoliths (25 × 25 × 10 cm) per subplot. Roots were then washed, air-dried and weighted.

One month before the end of the 4th cropping season, before the end of the rains, earthworms and nematodes were sampled. Earthworms were hand-sorted from two soil monoliths (25 × 25 × 10 cm) per subplot; they were counted and identified at a species level in the field and put back in the soil. Regarding soil nematodes, two soil samples (0–10 cm) were taken per subplot. Nematodes were extracted in the laboratory, from 100 g fresh soil, using a modified Baermann funnel method [37]. After 48 h of active filtering, nematodes in water were collected and counted using a stereomicroscope.

Here we focus on plant results obtained after the third cropping season in order to best perceive the combined effects of earthworm inoculation and cropping systems. This is also the year when crops were not disturbed by cattle trampling or climatic events.

## 2.5. Statistical Analyses

Data analyses were performed using the R statistical software [38] with a *p*-value threshold set at 10%. Prior to analyzing the effects of earthworm inoculation and cropping system on soil and plant properties, we first tested the soil homogeneity at the beginning of the experiment. Thus, initial soil properties were submitted to a one-way analysis of variance (ANOVA) (plot position as factor). A highly significant (*p*-value < 0.001) (Table 1) effect of plot position was observed for soil chemical variables (total C, total N and Pi). Due to the soil heterogeneity, we tested the main effects (earthworm inoculation and cropping system) on soil properties at the end of cropping seasons by using analysis of covariance (ANCOVA) in which covariates are initial soil properties. To determine the direction of significant effects of ANCOVA, we used multiple comparison tests based on estimated marginal means (hereafter emmeans, package emmeans) with Tukey adjustment.

**Table 1.** Mean ± SE and one-way ANOVA statistics for soil variables measured at the beginning of the experiment (2013). Treatments are abbreviated as follows: EW0: treatments where earthworms will not be inoculated, EW+: treatments where earthworms will be inoculated. Significant *p*-values are in bold.

	Units	Treatments		One-Way ANOVA
		EW0	EW+	Plot Position
Total C content	g kg <sup>-1</sup>	16.8 ± 0.6	14.3 ± 0.6	<b>0.005</b>
Total N content	g kg <sup>-1</sup>	1.84 ± 0.09	1.56 ± 0.05	<b>&lt;0.001</b>
Pi content	mg kg <sup>-1</sup>	10.65 ± 0.83	4.33 ± 0.55	<b>&lt;0.001</b>
Total C stock	Mg ha <sup>-1</sup>	19.3 ± 0.8	16.7 ± 0.7	<b>0.028</b>
Total N stock	Mg ha <sup>-1</sup>	2.11 ± 0.11	1.83 ± 0.08	<b>0.011</b>
Pi stock	kg ha <sup>-1</sup>	12.1 ± 0.9	5.1 ± 0.7	<b>&lt;0.001</b>
Bulk density	g cm <sup>-3</sup>	1.14 ± 0.04	1.18 ± 0.04	<b>0.058</b>

Regarding the changes in soil properties with time, the difference between mean final soil properties and mean initial soil properties was calculated. The obtained differences can be positive (meaning a gain) or negative (meaning a loss). For instance, difference in soil carbon content was calculated as:  $\Delta C$  (in g kg<sup>-1</sup>) = final C content – initial C content. This was calculated in the same way for P content and C and P stocks.

For agronomic parameters and soil biological properties (earthworm and nematode densities), the absence of significant effect of covariates on physico-chemical soil properties observed with ANCOVA (Table 2) led us to realize a three-way ANOVA (earthworm inoculation, cropping system, block) on the whole data. This analysis was followed by a Tukey HSD (honestly significant difference) post hoc test for multiple mean comparison. The normality of the data was verified for all ANOVA models on residuals using the Shapiro-Wilk test.

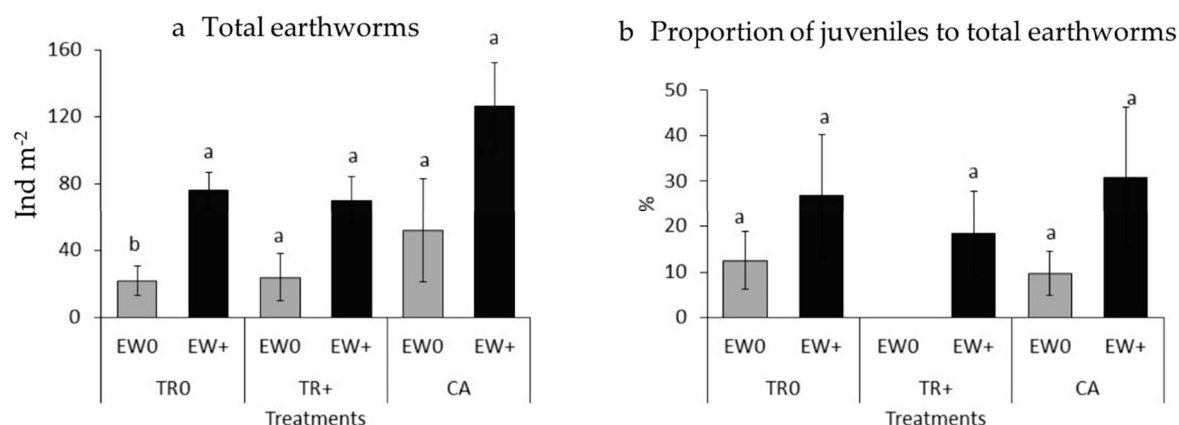
**Table 2.** Mean  $\pm$  SE for soil variables measured at the end of the 3rd cropping season (2016). Treatments are abbreviated as follows: TR0: traditional tillage without rice residues restitution, TR+: traditional tillage with rice residues restitution, CA: conservation agriculture system; EW0: without earthworm inoculation, EW+: with earthworm inoculation.

Soil Variables	Units	Treatments					
		TR0		TR+		CA	
		EW0	EW+	EW0	EW+	EW0	EW+
Chemical soil variables							
Total C content	$\text{g kg}^{-1}$	$23.7 \pm 1.1$	$22.6 \pm 0.6$	$24.3 \pm 1.0$	$22.7 \pm 1.4$	$23.6 \pm 0.6$	$22.2 \pm 1.0$
NH <sub>4</sub> <sup>+</sup> content	$\text{g kg}^{-1}$	$4.18 \pm 0.83$	$4.60 \pm 0.51$	$4.41 \pm 0.43$	$4.14 \pm 0.67$	$4.38 \pm 0.54$	$5.07 \pm 0.28$
Pi content	$\text{mg kg}^{-1}$	$11.86 \pm 1.90$	$5.70 \pm 1.93$	$13.36 \pm 1.56$	$8.99 \pm 2.64$	$14.38 \pm 0.90$	$9.73 \pm 2.02$
Total C stock	$\text{Mg ha}^{-1}$	$29.7 \pm 1.8$	$28.1 \pm 1.0$	$30.0 \pm 1.4$	$28.6 \pm 1.9$	$29.3 \pm 1.3$	$28.1 \pm 1.6$
Total NH <sub>4</sub> <sup>+</sup> stock	$\text{Mg ha}^{-1}$	$5.21 \pm 0.99$	$5.70 \pm 0.59$	$5.44 \pm 0.51$	$5.20 \pm 0.84$	$5.41 \pm 0.64$	$6.42 \pm 0.41$
Pi stock	$\text{kg ha}^{-1}$	$14.9 \pm 2.5$	$7.1 \pm 2.5$	$16.5 \pm 2.0$	$11.3 \pm 3.3$	$17.8 \pm 0.9$	$12.4 \pm 2.7$
Physical soil variables							
Bulk density	$\text{g cm}^{-3}$	$1.25 \pm 0.02$	$1.24 \pm 0.01$	$1.23 \pm 0.01$	$1.26 \pm 0.01$	$1.24 \pm 0.03$	$1.27 \pm 0.02$
Macroaggregates	%	$17.3 \pm 0.7$	$30.0 \pm 2.3$	$18.5 \pm 0.8$	$24.2 \pm 2.0$	$16.3 \pm 1.5$	$30.2 \pm 2.3$

### 3. Results

#### 3.1. Soil Biological Properties

At the rainy period of the 4th cropping season, ANOVA results showed that earthworm inoculation significantly increased the total density of *P. corethrurus* (sum of adults and juveniles) ( $p$ -value = 0.002). On average, it was three-fold higher in EW+ treatments than in EW0 treatments ( $91 \pm 11$  (individuals)  $\text{ind m}^{-2}$  vs.  $32 \pm 12$   $\text{ind m}^{-2}$ , respectively). We noticed that the proportion of the density of juveniles to the density of total earthworms was higher in EW+ (25%) than in EW0 (7%) (Figure 2). Regarding nematode density, we found no significant effect of earthworm inoculation ( $p$ -value = 0.106) despite higher values in EW+ ( $4.07 \pm 0.71$   $\text{ind g}^{-1}$  soil) than in EW0 ( $2.84 \pm 0.45$   $\text{ind g}^{-1}$  soil).



**Figure 2.** Earthworm density ( $\text{ind m}^{-2}$ ) at the end of the rainy period of the 4th cropping season according to treatments (earthworm inoculation, cropping system) (a) and proportion of juveniles to total earthworms (b). Error bars indicate standard errors. Letters indicate significant differences between EW0 and EW+ for different cropping systems according to Student t-test. See Figure 1 for legend.

For the cropping system factor, we observed that the density of earthworms tended to be significantly increased ( $p$ -value = 0.064) in CA treatments ( $89 \pm 23$  ind  $m^{-2}$ ) compared to tillage systems ( $49 \pm 12$  ind  $m^{-2}$  for TR0 and  $47 \pm 13$  ind  $m^{-2}$  for TR+) (Figure 2). However, ANOVA results showed no significant effect on nematode density, with  $p$ -value of 0.611. Additionally, the interaction of earthworm inoculation and cropping system had no influence on earthworm ( $p$ -value = 0.740) and nematode ( $p$ -value = 0.391) densities.

### 3.2. Soil Physico-Chemical Properties

#### 3.2.1. Soil Properties at the Beginning of the Experiment

ANOVA showed that plot position had a significant effect on initial soil properties, despite soil homogenization before cultivation (Table 1). Plots without earthworm inoculation (lower parts of the four blocks) had significant higher total C (+17.5%), total N (+17.9%), and Pi (+146%) contents, compared to plots where earthworms were inoculated. ANOVA also showed an effect of blocks, block 1 showing significantly lower N contents and stocks compared to other blocks (data not shown).

#### 3.2.2. Soil Properties at the End of the 3rd Cropping Season

At the end of the 3rd cropping season, we found that soil total C,  $NH_4^+$ , and Pi contents in EW0 were higher compared to values measured in EW+, with an average increase of 3%, 7%, and 29%, respectively (Table 2). However, according to ANCOVA results, the difference between earthworm treatments was not significant (Table 3). Additionally, total C,  $NH_4^+$ , and Pi stocks were also not affected by cropping system and initial soil characteristics. A significant block effect was found on total C content and stock. Regarding soil physical properties, the earthworm inoculation had no significant effect on bulk density ( $p$ -value = 0.214). Conversely, ANOVA showed that soil macroaggregates were significantly affected by earthworm inoculation ( $p$ -value < 0.001) (Table 3). In EW+, the proportion of soil macroaggregates to the total soil was 28%, while it was 17% in EW0 (Table 2). The cropping system had no significant effect on soil macroaggregates and bulk density, with  $p$ -values of 0.384 and 0.702, respectively (Table 3).

**Table 3.**  $p$ -Values of main effects (earthworm inoculation, cropping system, block) and their interaction and covariates (initial soil properties) on soil physico-chemical variables. ANCOVA and ANOVA were processed respectively for soil chemical and physical variables. Significant  $p$ -values are in bold.

Factors	Chemical Soil Variables						Physical Soil Variables	
	Total C Content	$NH_4^+$ Content	Pi Content	Total C Stock	$NH_4^+$ Stock	Pi Stock	Soil Macroaggregates	Bulk Density
<b>Main effects</b>								
Earthworm inoculation (E)	0.397	0.216	0.276	0.708	0.281	0.225	<b>&lt;0.001</b>	0.214
Cropping system (S)	0.840	0.643	0.632	0.827	0.466	0.704	0.384	0.702
Block	<b>0.029</b>	<b>0.066</b>	0.255	<b>0.031</b>	0.128	0.166	0.490	0.077
<b>Interaction</b>								
E*S	0.878	0.620	0.966	0.963	0.433	0.954	<b>0.063</b>	0.423
<b>Covariates</b>								
Initial total C content	0.999	0.229	0.801					
Initial total N content	0.210	0.325	0.417					
Initial Pi content	0.289	0.729	0.276					
Initial total C stock				0.756	0.141	0.393		
Initial total N stock				0.336	0.378	0.278		
Initial Pi stock				0.240	0.602	0.148		

#### 3.2.3. Changes in Soil Properties with Time

Earthworm inoculation had no significant effect on the difference between C content at the 3rd cropping season and the initial C content ( $\Delta C$  content) ( $p$ -value = 0.135, Tables 4 and 5). The same pattern was observed for  $\Delta C$  stock with a  $p$ -value of 0.218. C stock changes were significantly modified by the cropping system, with a higher C stock increase for

conservation agriculture than for tillage systems:  $\Delta C$  stocks were  $13.33 \pm 1.42 \text{ Mg ha}^{-1}$  in CA,  $9.06 \pm 1.29 \text{ Mg ha}^{-1}$  in TR+ and  $9.63 \pm 0.79 \text{ Mg ha}^{-1}$  in TR0 ( $p$ -value = 0.056). With regards to Pi stock changes, neither earthworm inoculation nor cropping system had a significant effect, with  $p$ -values of 0.659 and 0.120, respectively.

**Table 4.** Mean  $\pm$  SE of changes of soil properties with time (2013 to 2016). Legend: see Table 2.

Soil Variables	Units	Treatments					
		TR0		TR+		CA	
		EW0	EW+	EW0	EW+	EW0	EW+
$\Delta C$ content	$\text{g kg}^{-1}$	$6.02 \pm 0.76$	$7.48 \pm 0.65$	$4.92 \pm 1.30$	$7.40 \pm 1.71$	$8.55 \pm 1.49$	$9.77 \pm 1.70$
$\Delta \text{Pi}$ content	$\text{mg kg}^{-1}$	$0.04 \pm 0.71$	$1.29 \pm 1.88$	$2.94 \pm 1.35$	$3.63 \pm 2.63$	$4.01 \pm 1.28$	$5.66 \pm 2.40$
$\Delta C$ stock	$\text{Mg ha}^{-1}$	$8.95 \pm 1.43$	$10.30 \pm 0.74$	$7.78 \pm 1.19$	$10.35 \pm 2.30$	$12.54 \pm 2.02$	$14.12 \pm 2.23$
$\Delta \text{Pi}$ stock	$\text{kg ha}^{-1}$	$1.12 \pm 1.29$	$1.91 \pm 2.51$	$4.54 \pm 1.66$	$4.92 \pm 3.38$	$6.23 \pm 1.31$	$3.20 \pm 0.28$

**Table 5.** Three-way ANOVA statistics ( $p$ -values) of changes of soil properties with time (2013 to 2016). Legend: see Table 2. Significant  $p$ -values are in bold.

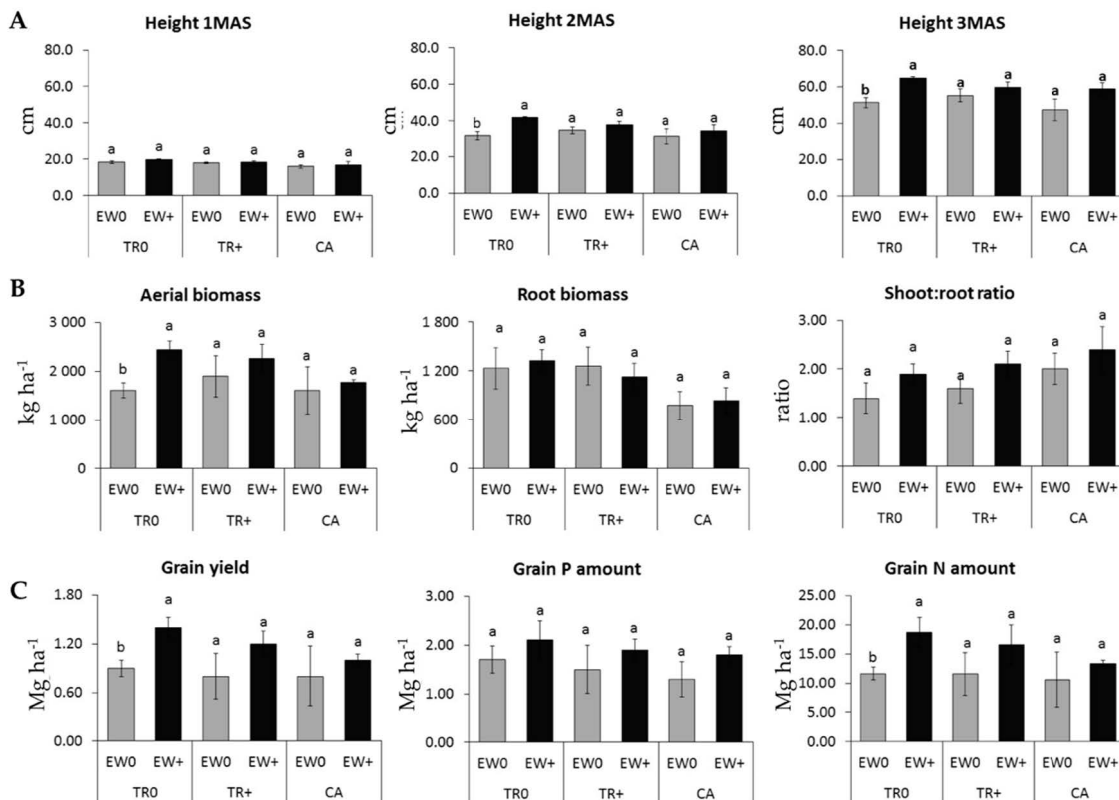
Soil Variables	Units	Three-Way ANOVA			
		Earthworm Inoculation (E)	Cropping System (S)	Block	E*S
$\Delta C$ content	$\text{g kg}^{-1}$	0.135	<b>0.089</b>	0.397	0.882
$\Delta \text{Pi}$ content	$\text{mg kg}^{-1}$	0.465	0.132	0.834	0.970
$\Delta C$ stock	$\text{Mg ha}^{-1}$	0.218	<b>0.056</b>	0.415	0.934
$\Delta \text{Pi}$ stock	$\text{kg ha}^{-1}$	0.659	0.120	0.709	0.971

### 3.3. Plant Parameters

#### 3.3.1. Rice Growth at the End of the 3rd Cropping Season

The effect of earthworm inoculation on rice height depends on the physiological stages: At the beginning of the tillering stage, there was no significant effect ( $p$ -value = 0.306), while at the end of the tillering stage and at the flowering stage, rice height was significantly higher in the presence of earthworms ( $p$ -value = 0.028 and  $p$ -value = 0.008, respectively). At the end of the flowering stage, rice height was 10 cm higher with earthworms than without ( $61.1 \pm 1.6 \text{ cm}$  and  $51.3 \pm 2.5 \text{ cm}$ , respectively) (Figure 3). Similarly, rice shoot biomass at the harvest significantly increased by 26% in treatments where earthworms were inoculated ( $p$ -value = 0.060). The shoot:root ratio was also significantly affected by earthworm inoculation ( $p$ -value = 0.091,  $2.62 \pm 0.64$  in EW+ and  $1.65 \pm 0.59$  in EW0 treatments) but not root biomass ( $p$ -value = 0.984). We observed a significant effect of the cropping system on rice height at the beginning of the tillering stage ( $p$ -value = 0.028) and non-significant effects at later stages ( $p$ -value = 0.351 at the end of the tillering stage,  $p$ -value = 0.403 at the flowering stage). At the beginning of tillering, rice height was lower in CA ( $16.3 \pm 0.9 \text{ cm}$ ) than in both TR treatments ( $18.9 \pm 0.4 \text{ cm}$  for TR0 and  $18.0 \pm 0.5 \text{ cm}$  for TR+). At the harvest, no significant effect of cropping system was found on shoot rice biomass ( $p$ -value = 0.300), but it was significant for root biomass ( $p$ -value = 0.094) with lower values in CA compared to tillage systems (Figure 3). Likewise, the shoot:root ratio was not significantly influenced by cropping system ( $p$ -value = 0.352).





**Figure 3.** Plant properties at the 3rd cropping season according to treatments (earthworm inoculation, cropping system). Error bars indicate standard errors. Letters indicate significant differences between EW0 and EW+ for different cropping systems according to Student t-test. (A) Plant height, (B) Plant biomass, (C) Plant production and nutrition. Legend: see Figure 1. MAS = month after sowing.

### 3.3.2. Rice Grain Yield and Nutrient Acquisition at the end of the 3rd Cropping Season

Statistical analyses showed that earthworm inoculation significantly affected the rice grain yield at the end of the third cropping season ( $p$ -value of 0.040, Table 6). We found that earthworm presence had a positive effect on rice yield (+44.6%). In EW+ treatments, rice grain yield was  $1.20 \pm 0.13 \text{ Mg ha}^{-1}$  while it was  $0.83 \pm 0.09 \text{ Mg ha}^{-1}$  in EW0. Similarly, rice nutrition was enhanced in treatments where earthworms were inoculated. Grain N and P amounts were respectively, significantly, increased by 43% and 28% in EW+ treatments compared to EW0 treatments (Table 6).

Regarding cropping system, rice grain yield tended to be lower in CA ( $0.88 \pm 0.15 \text{ Mg ha}^{-1}$ ) than in tillage systems ( $1.18 \pm 0.12 \text{ Mg ha}^{-1}$  for TR0 and  $1.04 \pm 0.17 \text{ Mg ha}^{-1}$  for TR+), but the difference was not significant ( $p$ -value = 0.281). Additionally, the interaction of earthworm inoculation and cropping system had no significant effect on any of the plant variables (Table 6).

**Table 6.** ANOVA *p*-values of main effects (earthworm inoculation, cropping system, block) and their interaction on plant variables at the 3rd cropping season (2016–2017). E\*S means the interaction between Earthworm inoculation (E) and Cropping system (S).

Plant Variables	Three-Way ANOVA			
	Main Effects			Interaction
	Earthworm Inoculation (E)	Cropping System (S)	Block	E*S
<i>Plant growth</i>				
Height 1MAS	0.306	<b>0.028</b>	<b>0.086</b>	0.771
Height 2MAS	<b>0.028</b>	0.351	0.713	0.375
Height 3MAS	<b>0.008</b>	0.403	0.983	0.477
Shoot biomass	0.126	<b>0.084</b>	0.268	0.280
Grain biomass	<b>0.047</b>	0.359	0.631	0.813
Shoot biomass	<b>0.060</b>	0.300	0.535	0.584
Root biomass	0.984	<b>0.094</b>	0.761	0.828
Total biomass	0.179	0.120	0.847	0.673
Shoot:root ratio	<b>0.091</b>	0.352	0.262	0.997
<i>Plant nutrition</i>				
Shoot N amount	0.680	0.310	0.184	0.314
Grain N amount	<b>0.037</b>	0.421	0.345	0.843
Aboveground N amount	<b>0.063</b>	0.486	0.271	0.714
Shoot P amount	0.140	0.547	0.311	0.671
Grain P amount	<b>0.056</b>	0.392	<b>0.065</b>	0.848
Aboveground P amount	0.167	0.637	<b>0.070</b>	0.781
<i>Plant production</i>				
Grain yield	<b>0.040</b>	0.281	0.668	0.802

## 4. Discussion

### 4.1. Success of Earthworm Inoculation

In our experiment, we did not eliminate native earthworms from the experimental field before earthworm inoculation. The earthworm density measured at the end of the experiment in the treatment without earthworm inoculation thus characterizes the earthworm population developing naturally in crop conditions. This density of 32 ind m<sup>-2</sup> is similar to other observations from upland agricultural fields in the Highlands of Madagascar, which also showed the dominance of the species *P. corethrurus* [31,39]. This value is half the density recommended by [25,40] (30 g m<sup>-2</sup>, i.e., 60 ind m<sup>-2</sup>) to observe a significant positive effect of earthworms on plant growth. With earthworm inoculation, we reached a density of 91 ind m<sup>-2</sup>, which is above this recommended value. There is no evidence regarding if this value results only from the last inoculation (Year 4), or from the inoculations of the four years of the experiment. The relatively high presence of juveniles in treatments EW+ may indicate that earthworms survive and develop well in this type of environment, especially when they are introduced at relatively high densities. These observations indicate evidence of earthworm activities throughout the experiment and prove the success of earthworm inoculation in upland agricultural field conditions in the Highlands of Madagascar. Survival and reproduction of introduced earthworms reinforce the interest of this inoculation technique. Conditions were thus reached to observe a positive effect of earthworms on the different plant and soil properties measured. Complementary studies should be realized to analyze whether inoculation should be practiced each year or whether initial inoculation during the first years result in the installation of a stable and abundant earthworm population. It was also interested to notice that earthworm density doubled in CA compared to tillage systems. This confirms the interest of such cropping systems without soil tillage, alternative to traditional ones, in increasing soil biota and especially earthworms [39,41–43]. The positive effect of the CA system is imputed to the reduction of soil tillage and the presence of plant cover, bringing organic matter and buffering climatic stresses.

### 4.2. Effects on Soil and Plant Properties

In our experiment, soil properties at the beginning of the experiment were variable, especially between subplots where earthworms were introduced and subplots without

earthworm inoculation. In that sense, it seems more relevant and interesting to focus on the changes in soil properties with time in the different plots.

We measured that C stock changes after 3 years ( $\Delta C$  stocks) were slightly more important in EW+ than in EW0, but this was not significant. This may indicate that our experiment was too short to observe a positive effect of earthworms on C storage as it has been observed in other experiments [44].

The inoculation of earthworms resulted in significant effects on plant properties at the third year of the experiment. Earthworms increased plant growth (height at the end of the tillering stage and at the flowering stage). The absence of effect on rice height at the beginning of the tillering stage may be explained by the short span (only one week) between earthworm inoculation and this measure of height. This highlights the importance of inoculating earthworms rapidly after sowing to observe rapid and positive effects on plant growth. The positive effect of earthworms was measured for shoot plant biomass (+26% in EW+ compared to EW0) and rice grain yield (+44.6%) as well. This fits with conclusions given by [12] in a meta-analysis, who gives an average increase in aboveground biomass of 23% and in yield of 25%. This increase is particularly visible for rice, which aboveground biomass strongly increased in the presence of earthworms compared to treatments without earthworms: +35% in Peru [21] and +42% in the review article by [40]. This positive effect of earthworms on plant growth and yield can be explained by different mechanisms [45]: increase in mineralization of organic matter [8] and higher availability of nutrients, especially N and P [2,34]; modification of soil porosity and aggregation improving water retention [46]; stimulation of microbial activity and production of auxin-like substances [47]; regulation of pests and pathogens [7,48]; stimulation of microbial plant symbiotes [45]; horizontal transfer of fertility through earthworm biomass decomposition [34]. In our experiment, we observed higher grain N and P amounts in the presence of earthworms, indicating higher availability of nutrients with earthworms. This is also confirmed by higher values of shoot:root ratio in the presence of earthworms, suggesting that plants are less constrained by nutrient acquisition and can invest less energy in roots and more energy in the aerial parts, following the theory of 'functional equilibrium' [49]. This biomass allocation towards aerial parts was observed in different studies (for instance [50,51]).

In contrast, cropping systems had no significant effects, neither on soil properties nor on plant growth, nutrition, or yield. We only observed a higher increase in C storage ( $\Delta C$  storage) in CA compared to tillage treatments, which was already described for Ferralsols of the Highlands of Madagascar [52]. These authors also observed an increase in P stock under CA aged 8 years old, which is longer than our experiment. This also confirms that it takes some years before the positive effects of CA on C and nutrient stocks become significant [53,54]. It was also noticed that the amount of crop residues in CA is important for defining the amplitude of CA effects on soil and plant properties. For instance, [55] noticed a significant positive effect of CA on weed pressure for a residue amount of 10 Mg ha<sup>-1</sup>. In our experiment, the residue amount was lower than this value (<5 mg ha<sup>-1</sup>).

## 5. Conclusions

In the Highlands of Madagascar, the inoculation of earthworms appears to be an efficient technology to improve both crop productivity (yield) and sustainability; sustainability seen here as the improvement of ecological functions and services such as increase in soil biota, C storage, and soil aggregation. The average surface of farms in this region is 0.9 ha, only 22% of the surface being used for upland cultivation, indicating small surfaces for upland fields. Earthworms of the species *P. corethrurus*, well known by farmers and locally called 'kanka-mena' (literally red worms) can be found easily, at high densities, in different places in the environment: around lowland fields, under trees, or under perennial grasses. It does not take much time to collect thousands of earthworms and to inoculate them in fields, at the beginning of the rainy season. This seems feasible with regards to the very small size of rain-fed rice plots in the family farms in the Highlands of Madagascar.

Inoculated earthworms survived at least during the whole rainy season and especially in CA situations characterized by no-tillage and rotation of rice with maize+dolichos. Inoculation resulted in positive effects on different functions and services, especially soil aggregation, rice nutrition, and rice yield (+44.6%). Studies giving such results in field conditions are relatively rare. Our study brings evidence that intensifying earthworm activity in cropped soils may increase upland rice yield and other agrosystem services such as C storage and erosion control.

**Author Contributions:** Conceptualization, O.R. and E.B.; methodology, O.R., E.B., M.R. (Malalatiانا Razafindrakoto), T.R. and M.R. (Michel Rabenarivo); validation, T.R., L.B., T.B. and J.T.; formal analysis, O.R., E.B. and M.R. (Malalatiانا Razafindrakoto); investigation, O.R., E.B. and M.R. (Malalatiانا Razafindrakoto); data curation, O.R., E.B. and M.R. (Malalatiانا Razafindrakoto); writing—original draft preparation, O.R. and E.B.; writing—review and editing, all authors; supervision, E.B.; project administration, E.B.; funding acquisition, E.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was partly funded by the project SECuRE (Soil ecological function restoration to enhance agrosystem services in rainfed rice cropping systems in agroecological transition), supported by the Agropolis Foundation under the reference ID 1605-007 through the “Investissements d’Avenir” program (Labex Agro: ANR-10-LABX-0001-01) under the framework I-SITE MUSE (ANR-16-IDEX-0006). The IRD (French Institute of Research for the Development) also contributed to the funding via the ERA-Net ARD II call (2012), CAMES Project (Impacts of Conservation Agriculture on Macrofauna diversity and related Ecosystem Services for improved farmer’s cropping systems and livelihoods in Highlands of Madagascar).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** We are thankful to Modeste Rakotondramanana, Marie-Paule Razafimanantsoa, and Koloïna Rahajarahilaza for technical help.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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