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1 Earthworms (*Millsonia anomala*, Megascolecidae) do not increase rice growth
2 through enhanced nitrogen mineralization.

3

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5

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8

9 **Abstract**

10

11 Earthworms have been shown to increase plant growth in 75% of the experiments
12 that have compared plant growth in their presence and absence. However, the
13 relative importance of the different mechanisms advanced to explain such a
14 stimulatory effect has never been tested. In a laboratory experiment we observed
15 increased growth of rice plants in the presence of earthworms (*Millsonia anomala*,
16 Megascolecidae) and demonstrated that enhanced nitrogen release (generally
17 considered as the principal mechanism involved in earthworm positive effect on
18 plants) was not responsible for this result: earthworms had the same stimulatory
19 effect on plant growth (+20 %) irrespective of whether the soil (provided with different
20 amounts of mineral-N fertilizer) was either N-limited or N-saturated. We discuss
21 alternative explanations for the observed variations in rice production

22

23

24 *Keywords:* Earthworm; Mechanisms affecting plant growth; Mineralization; Nitrogen
25 gradient

26

27

28 **1. Introduction**

29

30 Among the mechanisms by which earthworms modify plant growth at the
31 individual or community levels (Scheu, 2003; Brown et al., 2004), five have been
32 claimed to be responsible for the positive effect noted on plant production: (i)
33 increased mineralization of soil organic matter, which increases nutrient availability
34 (Barois et al., 1987; Knight et al., 1989; James, 1991; Curry and Byrne, 1992; Lavelle
35 et al., 1992; Subler et al., 1997), especially for nitrogen (N), the major limiting nutrient
36 in terrestrial ecosystems; (ii) modification of soil porosity and aggregation (Blanchart
37 et al., 1999; Shipitalo and Le Bayon, 2004), which induces changes in water and
38 oxygen availability for plants (Doube et al., 1997; Allaire-Leung et al., 2000); (iii)
39 production of plant growth regulators via the stimulation of microbial activity
40 (Frankenberger and Arshad, 1995; Muscolo et al., 1998; Nardi et al., 2002;
41 Quaggiotti et al., 2004); (iv) biocontrol of pests and parasites (Stephens et al., 1994;
42 Clapperton et al., 2001; Blouin et al., 2005); (v) stimulation of symbionts (Gange,
43 1993; Pedersen and Hendriksen, 1993; Furlong et al., 2002).

44

45 Enhanced N mineralization is the best documented mechanism and is generally
46 thought to be the most important. However, despite 313 studies on earthworm effects
47 on plant growth, (Brown et al., 1999; Scheu, 2003), no attempts have been made to
48 assess the relative importance of each of these potential mechanisms (Scheu, 2003;
49 Brown et al., 2004). Here, in a particular experimental case, we evaluate the
50 importance of enhanced mineralization by growing rice fertilised with different levels

51 of mineral N. If enhanced N mineralization is the main mechanism involved in the
52 stimulatory effect of earthworms on plant growth, earthworm effect should be most
53 important when the N availability in soil is low since earthworm activities provide the
54 major amounts of mineral N. Under the same hypothesis, the stimulatory effect of
55 earthworm activities should disappear when the availability of mineral N in the soil is
56 high, the N surplus due to earthworms becoming negligible.

57

58

59 **2. Materials and Methods**

60

61 *2.1 Experimental units*

62

63 Young rice seedlings (*Oryza sativa*, cv. Moroberekan) were grown for three
64 months under a 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ artificial light source, at 28 °C/day and 24
65 °C/night temperatures and at 75 % +/- 5 % air moisture. Pots (10 cm in diameter)
66 were filled with 1 kg of a sandy ultisol from Lamto savannah (Ivory Coast). Nitrogen is
67 particularly limiting in the soil of the Lamto savannah : 500 mg kg^{-1} total N (Abbadie
68 and Lensi, 1990; Lensi et al., 1992; Gilot, 1997; Lata et al., 1999), 1.5 to 7.5 mg kg^{-1}
69 NH_4^+ and < 1 mg kg^{-1} NO_3^- (Martin, 1990).

70

71 *2.2 Earthworms*

72

73 The original soil fauna was eliminated by sieving (2 mm mesh) and freezing the
74 soil (Blouin et al., 2005). The earthworm *Millsonia anomala*, (Omodeo and Vaillaud,
75 1967), endemic to this region, is a large mesohumic compacting species (Blanchart

76 et al., 1999), whereas the other species, *Chuniodrilus zielae*, (Omodeo and Vaillaud,
77 1967) has a somewhat larger geographical distribution and is a thin polyhumic
78 decomposing worm (Blanchart et al., 1999). These two endogeic earthworms feed
79 on soil organic matter; consequently, the soil was not amended with organic matter.

80 Three immature *M. anomala* (0.85 ± 0.20 g biomass) and three *C. zielae* ($0.07 \pm$
81 0.02 g) were added in each pot according to the treatments. The population density
82 of *M. anomala* was similar to that of natural populations, whereas that of *C. zielae*
83 was slightly lower (Lavelle, 1978). There were four treatments: *M. anomala* alone
84 (M), *C. zielae* alone (Ch), both species present (MCh), and a control without
85 earthworms (C).

86 Some earthworms died during the experiment but the mortality of *M. anomala* and
87 *C. zielae* had no significant effect on total ($P = 0.24$ and 0.14 respectively),
88 aboveground ($P = 0.34$ and 0.12 respectively) or belowground ($P = 0.14$ and 0.21
89 respectively) dry biomasses. The mortality was quite similar in all treatments and not
90 related to the N concentrations.

91

92 *2.3 Fertilizer*

93

94 Soil mineral N content was increased by watering daily rice plants with a complete
95 fertilizing solution in which all major mineral nutrients and oligo-elements were kept at
96 constant concentrations except N. Since NH_4^+ is the preferred form of nitrogen taken
97 up by rice (Fried et al., 1965; Sasakawa and Yamamoto, 1978), we modified the
98 Hoagland-Arnon solution by replacing $\text{Ca}(\text{NO}_3)_2$ with CaCl_2 and KNO_3 with
99 $\text{NH}_4(\text{SO}_4)_2$ and adding silicium which is necessary for many grasses. Therefore, the
100 composition of the fertilizer was KH_2PO_4 : $2939 \mu\text{mol l}^{-1}$; CaCl_2 : $2495 \mu\text{mol l}^{-1}$; MgSO_4 :

101 3950 $\mu\text{mol l}^{-1}$; Na_2SiO_3 : 996 $\mu\text{mol l}^{-1}$; Fe-EDTA (13 % of Fe): 5 mg l^{-1} ; Oligo-
102 elements : H_3BO_3 : 55 $\mu\text{mol l}^{-1}$; MnSO_4 : 20 $\mu\text{mol l}^{-1}$; ZnSO_4 : 0,6 $\mu\text{mol l}^{-1}$; Na_2MoO_4 : 0,4
103 $\mu\text{mol l}^{-1}$; CuSO_4 : 0,6 $\mu\text{mol l}^{-1}$. NH_4^+ was supplied at five different concentrations (0,
104 25, 100, 400, 1600 $\mu\text{mol l}^{-1}$) in each of the four fauna treatments, resulting altogether
105 in 20 treatments; each was replicated 3 times.

106

107

108 *2.4 The NH_4^+ gradient*

109

110 The test of our hypothesis requires a range of N concentrations, from deficient to
111 excess N availability. To ensure N-limitation for plant production in the 0 $\mu\text{mol l}^{-1}$ NH_4^+
112 treatment, we used a soil that only had 0.05 % total N content (Martin, 1990; Abbadie
113 and Lensi, 1990; Lensi et al., 1992; Gilot, 1997; Lata et al., 1999) and we added all
114 the other macro- and micronutrients at adequate concentrations.

115 NH_4^+ is known to be limiting to rice seedlings growth below 500 $\mu\text{mol l}^{-1}$ in
116 hydroponical conditions (Wang et al., 1993; Kirk, 2001). In our experiments, the 1600
117 treatment showed clear evidence of NH_4^+ toxicity : excessive N concentration in plant
118 tissues (fig. 1 a) and deficit in root production (Britto and Kronzucker, 2002) (fig. 1 b).
119 This argues in favour of an alleviation of the N-limitation in the 1600 $\mu\text{mol l}^{-1}$
120 treatment.

121

122 Fig. 1

123

124

125 *2.4 Statistical analysis*

126

127 Two complementary analyses were conducted on our data set: an ANOVA and
128 an ANCOVA. We checked the homogeneity of variances (Bartlett's test) and
129 normality of the residues (Shapiro-Wilk's test) for total, aboveground and
130 belowground biomasses. Residuals met the conditions of homoscedasticity ($P =$
131 0.43, 0.69, 0.20 respectively) and normality ($P = 0.80, 0.67, 0.22$ respectively). With
132 full ANOVA model, the effects of nitrogen, *M. anomala*, *C. zitelae* and the interactions
133 between these factors were tested (Table 1). In this analysis, nitrogen was
134 considered as a discrete factor. To visualize our results more easily, an ANCOVA
135 was conducted considering nitrogen as a continuous variable. The effect of nitrogen
136 was modelled using a polynom whose order is statistically determined. The
137 significant factor(s) of the ANOVA (earthworm species) may affect significantly some
138 of the parameters of the polynom, modifying the regression equation as compared
139 with the control one.

140

141

142 **3. Results**

143

144 As expected, the ANOVA showed that the N gradient had a significant effect on
145 plant dry total and above-ground biomasses (Table 1). Comparisons between means
146 (Tukey's test for multiple comparisons) showed that total and above-ground
147 biomasses were not significantly different from the $0 \mu\text{mol l}^{-1}$ to the $100 \mu\text{mol l}^{-1}$
148 treatments, but increased significantly from the 100 to the $400 \mu\text{mol l}^{-1}$ treatments
149 (respectively +18 and 19.5 %) and from the 400 to the $1600 \mu\text{mol l}^{-1}$ treatments
150 (respectively +19 and 31 %), showing that plant growth was N-limited at the lowest

151 NH_4^+ concentrations. No significant effect of the N gradient was observed on below-
152 ground biomass (Fig. 1c). *M. anomala* had a significant effect on total, above-ground
153 and below-ground biomasses. However, its interaction with the N concentration was
154 not significant (Table 1). The positive effect of *M. anomala* on plant production did not
155 depend on the N concentration. Nor *C. zielae* neither its interactions with the N
156 concentration or *M. anomala* had a significant effect on plant production (Table 1).

157

158 Table 1

159

160 As *C. zielae* had no significant effect in the ANOVA, we realized the ANCOVA to
161 test the effect of *M. anomala*, independently of the presence of *C. zielae* : control (C)
162 and *C. zielae* treatments, and *M. anomala* and *M. anomala-C. zielae* treatments
163 respectively were pooled together.

164

165 Table 2

166

167 The relationship between plant total and above-ground biomasses with the N-
168 gradient fitted on a second order polynomial ($y = a + bx + cx^2$) (Table 2). Below-ground
169 biomass did not vary significantly along the N-gradient ($y = a$). For total and above-
170 ground biomasses *M. anomala* had a significant positive effect on the parameter a,
171 but not on b or c (Table 2; Fig. 2a and 2b). *M. anomala* also had a significant effect
172 on the parameter a for below-ground biomass (Fig. 2c). Taken together, *M. anomala*
173 had a constant positive effect on plant production along the N-gradient : + 20, 16 and
174 35 % for the total, above-ground and below-ground biomasses respectively.

175

176 Fig. 2

177

178 4. Discussion

179

180 We postulated that if enhanced N mineralization is an important mechanism
181 involved in the positive effect of earthworms on plant growth, earthworms should
182 have a positive effect in a N-limited environment but not in a N-saturated
183 environment, where the enhanced mineralization of N is negligible. Both an ANOVA,
184 taking into account the presence of *C. zitelae*, and an ANCOVA, where the presence
185 of *M. anomala* was considered independently of the presence of *C. zitelae*, showed
186 that the effect of *M. anomala* was constant whatever the N concentrations. Thus, we
187 can reject the hypothesis that the main effect of *M. anomala* on plant production was
188 due to increased N mineralization. If *M. anomala* would increase plant production
189 through an enhanced mineralization of other nutrients (such as P, K... etc), this
190 would have produced no effect on plant in a N-limited environment, but an increase
191 in plant growth in situations where plant is no longer limited by N. This was not
192 observed in our experiment (Fig. 2) and we therefore reject this hypothesis.

193

194 In contrast to *M. anomala*, *C. zitelae* had no significant effect on plant biomass.
195 Only a slight positive effect of *C. zitelae* on root biomass had been observed
196 previously (Derouard et al., 1997). In our experiment the soil was probably not
197 compacted enough to allow the decompacting effect of *C. zitelae* to influence plant
198 growth significantly.

199

200 The increase in plant aboveground biomass simultaneously with the absence of
201 an increase in belowground biomass along the N gradient indicates that the rice
202 plants allocated less resource to the root system as N availability increased
203 (Thornley, 1972; Wilson, 1988; Andrews, 1993). In contrast, the presence of *M.*
204 *anomala* increased both above- and belowground biomasses. Two separate
205 mechanisms were probably responsible for the differing plant responses to the N
206 gradient and to the presence or *M. anomala* (see below).

207 Since *M. anomala* effect was constant over the N-gradient, our experiment does
208 not support the widespread belief that enhanced mineralization by earthworms is the
209 main causal mechanism to explain the increased rice growth in presence of *M.*
210 *anomala*. Nevertheless, this mechanism could be important in other plant-earthworm
211 associations. Moreover, our experiment allows the hypothesis of an enhanced
212 nitrogen mineralization to be excluded at high levels of N, but not at low levels:
213 different mechanisms could in fact be involved at different N concentrations. The
214 mechanism(s) responsible for the positive effect on plant growth at high N
215 concentration cannot be clearly identified from our results. Nevertheless, an
216 improvement of soil water or oxygen availabilities by *M. anomala* can probably be
217 ruled out because soil was maintained at an optimal value of 80 % field capacity by
218 daily watering. Control of specialist parasites or the stimulation of symbionts likely did
219 not occur in our experiment because the soil originated from a non-cultivated
220 savannah with no crop-specific parasites or symbionts, and the original soil fauna
221 had been eliminated. We cannot, however, discard the hypothesis that microbial
222 generalist parasites or symbionts may have been controlled or stimulated by *M.*
223 *anomala*. This leaves the production of plant growth regulators (Frankenberger and
224 Arshad, 1995; Muscolo et al., 1998; Nardi et al., 2002; Quaggiotti et al., 2004) as the

225 probable explanation of the stimulatory effect of *M. anomala* on rice in our
226 experiment. The possibility and the detailed mechanisms of the control of plant
227 physiology via phytohormones secreted into soils by the bacteria associated with
228 earthworms activities should be studied thoroughly.

229

230

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232

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237

238

239

240

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242

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362

363 Table 1

364 Factors affecting total, shoot and root biomasses from a three-way ANOVA

	df	biomass								
		total			aboveground		belowground			
		F	P		F	P	F	P		
Nitrogen	4	8.5	<0.0001	***	18.3	<0.0001	***	0.8	0.53	
<i>M. anomala</i>	1	9.1	0.004	**	8.4	0.006	**	9.4	0.004	**
<i>C. zielae</i>	1	1.0	0.33		1.1	0.29		0.7	0.39	
Nitrogen * <i>M. anomala</i>	4	0.9	0.50		0.9	0.45		0.7	0.63	
Nitrogen * <i>C. zielae</i>	4	0.9	0.49		0.4	0.78		1.8	0.16	
<i>M. anomala</i> * <i>C. zielae</i>	1	0.2	0.64		0.2	0.64		0.2	0.66	
Nitrogen * <i>M.a.</i> * <i>C.z.</i>	4	1.3	0.28		1.1	0.37		1.7	0.17	

365 ***: p < 0.001, **: p < 0.01, *: p < 0.1, n = 60

366

367 Table 2

368 Polynomial equations determined with the ANCOVA

model: $y = a + bN + cN^2 + dN^3$ coefficients of	df	biomass								
		total			aboveground		belowground			
		F	P		F	P	F	P		
N (b)	1	18.0	<0.0001	***	42.0	<0.0001	***	0.2	0.68	
N ² (c)	1	14.5	0.0004	***	33.1	<0.0001	***	0.2	0.64	
N ³ (d)	1	0.03	0.87		0.98	0.33		1.3	0.25	
<i>M. anomala</i> (a)	4	8.7	0.005	**	8.27	0.006	**	8.5	0.005	**
N * <i>M. anomala</i> (b)	4	0.3	0.57		0.26	0.61		0.4	0.53	
N ² * <i>M. anomala</i> (c)	1	0.001	0.98		0.0004	0.99		0.01	0.91	
N ³ * <i>M. anomala</i> (d)	1	0.8	0.37		1.19	0.28		0.3	0.58	

369 ***: p < 0.001, **: p < 0.01, *: p < 0.1, n = 60

370 Fig. 1

371 Rice responses to the N-gradient according to the presence of earthworms. (a)
372 Percentage of N in plants. (b) Shoot:root ratio of plants. Without lines: control without
373 fauna; Horizontal lines: *M. anomala*; Vertical lines: *C. zielae*; Horizontal and vertical
374 lines: *C. zielae* and *M. anomala*. Means \pm s.e., n = 60.

375

376 Fig. 2

377 Response of plant dry biomasses to earthworms along a nitrogen gradient. (a)
378 Total biomass, (b) Shoot biomass, (c) Root biomass. Nitrogen concentration was
379 expressed as $\log(1+[N])$ for more clarity. The polynomial equation of curves was
380 obtained by regression analysis, where the significance of the different coefficients
381 was tested. O: control without fauna; Δ : *C. zielae*; +: *M. anomala*; x: *C. zielae* and *M.*
382 *anomala*. The different regression lines are represented only for the significant
383 factors; solid line: without *M. anomala*, dashed line: with *M. anomala*. n = 60.

384

385