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

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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 :  $500 \text{ mg kg}^{-1}$  total N (Abbadie  
68 and Lensi, 1990; Lensi et al., 1992; Gilot, 1997; Lata et al., 1999),  $1.5 \text{ to } 7.5 \text{ mg kg}^{-1}$   
69  $\text{NH}_4^+$  and  $< 1 \text{ mg kg}^{-1} \text{NO}_3^-$  (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* ( $0.85 \pm 0.20$  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  $\text{NH}_4^+$  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  $\text{CaCl}_2$  and  $\text{KNO}_3$  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  $\text{KH}_2\text{PO}_4$ :  $2939 \mu\text{mol l}^{-1}$ ;  $\text{CaCl}_2$ :  $2495 \mu\text{mol l}^{-1}$ ;  $\text{MgSO}_4$ :

101 3950  $\mu\text{mol l}^{-1}$ ;  $\text{Na}_2\text{SiO}_3$ : 996  $\mu\text{mol l}^{-1}$ ; Fe-EDTA (13 % of Fe): 5  $\text{mg l}^{-1}$ ; Oligo-  
102 elements :  $\text{H}_3\text{BO}_3$ : 55  $\mu\text{mol l}^{-1}$ ;  $\text{MnSO}_4$ : 20  $\mu\text{mol l}^{-1}$ ;  $\text{ZnSO}_4$ : 0,6  $\mu\text{mol l}^{-1}$ ;  $\text{Na}_2\text{MoO}_4$ : 0,4  
103  $\mu\text{mol l}^{-1}$ ;  $\text{CuSO}_4$ : 0,6  $\mu\text{mol l}^{-1}$ .  $\text{NH}_4^+$  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 $\text{NH}_4^+$ 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  $\mu\text{mol l}^{-1}$   $\text{NH}_4^+$   
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  $\text{NH}_4^+$  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  $\text{NH}_4^+$  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  $\text{NH}_4^+$  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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362



363 Table 1

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

	dl	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	dl	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 <sup>2</sup> (c)	1	14.5	0.0004	***	33.1	<0.0001	***	0.2	0.64	
N <sup>3</sup> (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 <sup>2</sup> * <i>M. anomala</i> (c)	1	0.001	0.98		0.0004	0.99		0.01	0.91	
N <sup>3</sup> * <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;  $\Delta$ : *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

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