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The Effect of Endogenous Earthworm Extracts (*Aporrectodea molleri*) on the Growth of Beneficial Soil Bacteria

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Abstract

Earthworms, which represent the first animal mass, play an important role in improving soil fertilization and biological properties of the rhizosphere. In the present investigation, the authors were interested in studying the post-mortem effect of this considerable biomass on the growth of soil bacteria. Therefore, the effect of crude extracts of freshly harvested earthworms (FHE) and of earthworms starved for 10 days (FE) on the growth of two bacterial strains, *Escherichia coli* and *Pseudomonas fluorescens* was investigated in vitro. The efficiency of these two extracts was compared at different concentrations with a conventional medium (NA). The results showed that the efficiency of FHE and FE extracts on bacterial growth was significantly ($P < 0.05$) greater than that of the NA medium. The optimal concentration of the extracts for *E. coli* and *P. fluorescens* was, respectively, 2 and 33.33 times lower than the conventional medium. Moreover, the growth rate at those concentrations was more than 3 times greater. Thus, the FHE and FE extracts would be richer in more diverse nutrients and growth factors. Furthermore, the efficiency of the FE extract was, at all concentrations, higher than the FHE extract, which proves that the nutrients are mainly endogenous to earthworms.

Keywords: earthworms, *Pseudomonas fluorescens*, fertility, soil, nutriments.

Резюме

Земните червеи играят важна роля за подобряване на почвеното плодородие и биологичните свойства на ризосферата. В настоящото изследване авторите се фокусират върху изучаването на ефекта на тази биомаса върху растежа на почвените бактерии. Ефектът на екстракти от прясно събрани земни червеи (FHE) и на земните червеи, гладували в продължение на 10 дни (FE) върху развитието на два бактериални щама, *Escherichia coli* и *Pseudomonas fluorescens* е изследван *in vitro.* Ефективността на тези два екстракта се сравнява при различни концентрации с конвенционална среда (NA). Резултатите показват, че ефективността на екстрактите от FHE и FE върху растежа на бактериите е значително по-голяма ($P < 0,05$) от тази на средата с NA. Оптималната концентрация на екстрактите за *E. coli* и *P. fluorescens* е съответно 2 и 33.33 пъти по-ниска от конвенционалната среда. Освен това, скоростта на растеж при тези концентрации нараства повече от 3 пъти. Това предполага, че екстрактите от FHE и FE са по-богати и по-разнообразни на хранителни вещества и растежни фактори. Ефективността на екстракт от FE е по-висока при всички концентрации от тази на екстракт от FHE, което доказва, че хранителните вещества са предимно ендогенни за земните червеи.

Introduction

The functioning of terrestrial ecosystem is highly dependent on bacterial activity. In fact, soil bacteria are effectively involved in the decomposition and matter recycling processes. In agriculture, plant growth-promoting rhizobacteria (PGPR) enhance the availability and uptake of nutrients by plants and limit the spread of pests (Sellan *et al*.,

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2020) mineral fertilizer (NPK. Soil bacteria interact with earthworms, which are the main soil animal biomass. This interaction is essential for the natural improvement of soil fertility (Wu *et al*., 2012). These burrowing animals are involved in the dynamics of bacterial populations and ensure, through the gut, their dispersion in the soil (Picón *et al*., 2015; Aira, 2018). Moreover, earthworms may affect bacterial populations directly by feeding and digestive processes or indirectly by burrowing and casting activities. Parle (1963) has already reported the richness of earthworm's excrement (casts) with microbial populations. Therefore, earthworms provide an ideal environment for enhanced activity levels and multiplication of bacteria.

Endogenic species of earthworms are much more likely to affect PGPR bacterial communities because they live, surrounding plant roots, in the rhizosphere matrix. In addition, they tend to build more permanent and deeper burrows, which maintain contact between beneficial soil bacteria and roots. Furthermore, the ability of earthworms to stimulate bacterial activity and increase bacterial populations depends greatly on the earthworm's special range of activity (Edward, 2004).

In this study, the post-mortem effect of earthworm cadaver decomposition on bacterial proliferation was investigated. In order to avoid any interference with soil nutrients, all experiments were carried out *in vitro*. The crude extracts of earthworms *Aporrectodea molleri* (endogenous earthworm) were prepared from 2 earthworm lots with the same dry weight: one was freshly harvested (FHE), the other was previously deprived of food and soil (FE). Starving the earthworms was necessary to ensure the removal of soil debris present in their digestive tract. From these two crude extracts, bacterial culture media were prepared at different concentrations. The efficiency of each extract was evaluated by, simultaneously, the relative concentration of soluble matter and the relative growth of bacteria.

Materials and Methods

Biological material

Earthworms: *A. molleri* was harvested in the region of Akrach about 20 km south-east of Rabat (Morocco). Bacteria: the strain *Escherichia coli* ATCC 25922 was loaned to us by the National Institute of Hygiene (INH) of Rabat (Morocco); the strain *Pseudomonas fluorescens* belongs to the collection of our laboratory: Earthworm, Improvement of soil productivity and Environment (LAPSE) of Rabat (Morocco). It was identified by sequencing the DNA gene corresponding to 16S rRNA in the National Centre for Scientific Research of Rabat (CNRST).

Preparation of crude earthworm extracts

The first lot of earthworms freshly harvested (FHE) of fresh weight: FW = 100g, was submersed in a container filled with pure methanol and placed in an oven (70°C). After the total evaporation of water and methanol, the lot of dry earthworms was ground into powder and then weighed to determine the dry weight (DW). The extraction was carried out in an oven at 70°C by maceration in successive baths of distilled water. In each maceration, the brown macerate (supernatant) was gently recovered, and then the maceration baths were continued in the same way until the final macerate became transparent. The successive macerates were accumulated and then doubly filtered; first on glass cotton and then on Whatman paper. The filtrate was evaporated in an oven (70°C) and then weighed to determine the dry weight. This dry residue, consisting entirely of water-soluble material, represented the crude extract of the FHE lot ($DW_{CE,\ FHE}$) from which culture media were prepared by successive dilutions in distilled water.

The second lot of earthworms (FE) was quickly rinsed with deionized water and then starved for 8 to 10 days in earthen pots without food or soil. At the end of the fast, the earthworms were quickly rinsed with deionized water and dried. The fresh weight of FE lot must be equal to that of FHE, i.e., FW = 100g. The crude extract of this 2nd lot of FE was prepared according to the same protocol as the crude extract of the first lot of FHE. As before, the dry weight of the FE (DW_{FE}) lot was determined as well as that of the soluble residue of its crude extract ($DW_{CE,\ FE}$).

Preparation of bacterial culture media (FHE and FE)

Eight g dry weight of crude earthworm extract from the first lot (FHE) and the second lot (FE) were weighed separately. Each quantity was dissolved in 200 mL of distilled water. The pH of the two solutions, which was acidic (pH=4), was adjusted to neutral pH at the same pH value as that of the conventional NA medium. This adjustment was necessary to discard the effect of pH on bacterial growth. The 200 mL of FHE and FE solutions were each divided into 2 volumes of 100 mL. The first volume (100 mL) constituted the C_1 culture

medium at 4%. The second volume (100 mL) was adjusted to 200 mL with distilled water. After homogenization, this volume was divided into two; the first volume constituted the C_2 culture medium at 2%, i.e., a dilution factor (Fd) equal to 2X. The 2nd remaining volume (100 mL) was diluted in the same way as above to obtain the C_3 medium at 1%; Fd = 4X. The series of successive dilutions continued in the same way for the two extracts solutions until the culture media C_4 was obtained at 0.5% (Fd = 8X), C_5 at 0.25% (Fd=16X), C_6 at 0.12% (Fd=32X), C_7 at 0.06% (Fd=64X) and C_8 at 0.03% (Fd=128X). The two series of culture media FHE and FE were used in solid form.

Preparation of conventional culture medium (NA)

The effect on bacterial growth of culture media (C_1, ..., C_8) based on earthworm extracts (FHE or FE) was compared to that of conventional medium: Nutrient Agar (NA). This conventional medium consisted of 2% of nutritive material assimilated at neutral pH.

Bacterial inoculation, growth parameters and efficiency parameters

A 10X dilution series was carried out from the *E. coli* and *P. fluorescens* suspensions in order to obtain between 30 and 300 colonies/dish. From the selected bacterial suspension, 0.1 mL was deposited and spread in solid medium. The Petri dishes were incubated for 24 hours at 37°C for *E. coli* and at 30 C for *P. fluorescens*. Bacterial growth was determined by the average number of colonies formed per dish (NCF). The efficiency of the FHE or FE extract ($E_{FHE\ or}\ E_{FE}$) was expressed compared to the conventional NA medium by two parameters:

- relative concentration (RC) - the concentration of the FHE or FE extract ($C_{FHE\ or\ FE}$) relative to the concentration of the NA medium (C_{NA})

$$RC = (C_{FHE\ ou\ FE}/C_{NA})\times 100$$

- relative growth (RG)- the growth in the FHE or FE extracts media ($G_{FHE\ ou\ FE}$) compared to growth in the conventional NA media (G_{NA}).

$$RG = (G_{FHE\ ou\ FE}/G_{NA})\times 100$$

The FHE or FE extract was said to be effective or more effective than the conventional NA medium when RC was less than or equal to 100% (Concentration of the extract was less than or equal to that of the NA medium) and RG was higher than or equal to 100% (Growth in extract was greater than or equal to that in NA medium). The FHE or FE extract was described as ineffective when RC and RG did not meet the efficiency conditions mentioned above. Optimal efficiency was designated when at the concentration of the FHE or FE extract, which was less than or equal to that of NA medium, a maximum growth greater than or equal to that of the NA medium was obtained.

Analysis of statistical data

The data concerning the average number of colonies formed (NCF) were submitted to ONE WAY ANOVA (SPSS software version 22) in accordance with the experimental models. Significant differences were determined at a probability level of 0.05 and Tukey's multiple comparison test was used to compare the means. All data were expressed as means ± standard error.

Results

Yield of earthworm crude extract

The preparation of the earthworm crude extracts (CE) according to the protocol indicated in this study made it possible to find interesting information. All of this information is presented in Table 1. The preparation of the crude extracts was carried out from two lots of earthworms with the same fresh weight (FW = 100g). The first lot was freshly harvested from the field (FHE). The second lot were deprived of food and soil for 10 days until elimination of the digestive content (FE).

Table 1. Summary of the data obtained during the preparation of the crude extracts of freshly harvested earthworms from lot (FHE) and previously deprived of soil and food (FE).

	Earthworm lots	
	FHE	FE
FW: Fresh weight (g)	100	100
DW: Dry weight (g)	25	19
FW-DW Water content (g or %)	75 (75%)	81 (81%)
DW_{FHE}-DW_{FE} Contents of the digestive tract (g or %)	6 (24%)	
DW_{CE} Dry weight of crude extracts (g or %)	11 (44%)	13 (68.4%)

The dry weight (DW) of the FHE and FE lots was 25 g and 19 g, respectively. The difference between fresh and dry weight (FW-DW) of the earthworm lots gave the water content expressed in grams or in percentages, either 75 g or (75%) for FHE, and 81 g or (81%) for (FE). The difference in water content between the two lots was related to the quantity, in dry weight, of solid detritus of the digestive content of FHE. This quantity was evalu-

ated by the difference of the dry weights of the two lots (DW_{FHE}-DW_{FE}), which was equal to 6 g, representing 24% of the dry weight of this lot. Finally, the quantity, in dry weight, of the crude extracts of earthworms (DW_{CE}), consisting only of water-soluble matter, was 11 g or 44% of the dry weight of the FHE lot and 13 g or 68.4% of the dry weight of the lot FE. It was noted that the FE lot contained more soluble matters than the FHE lot.

Effect of freshly harvested earthworm extract (FHE) on bacterial growth

The effect of the crude extract FHE at different concentrations C_n (C_1 = 4%, ..., C_8 = 0.03%) on the growth of *E. coli* and *P. fluorescens* gave the results presented in Table 2. For the *E. coli* strain, the average number of colonies formed increased as the concentration of the FHE extract decreased, to reach a growth of NCF = 181 colonies/dish at concentration C_3=1%.

The growth then gradually decreased below C_3, until reaching a low value of 42 colonies/dish at concentration C_8 = 0.03%. Also, the growth decreased significantly at concentrations above C_3. The effectiveness of the FHE extract compared to the conventional NA medium (E_{FHE}) was determined by simultaneously comparing the relative concentration RC, which had to be less than or equal to 100% and by the relative growth RG, which had to be greater than or equal to 100%. In the effective zone between C_3=1% to C_7=0.O6%, the relative RC concentration of FHE varied from 50% to only 3% of that of NA media. The relative RG growth ranged from 321.32% to 97.04%. Optimal efficiency was observed at C_3=1%, where growth was maximum (NCF=181 colonies/dish). At this concentration, representing only half that of the NA media (RC=50%), the relative growth was RG=321.32%. The NCF at concentrations C_1, C_2 and C_8 was significantly different and lower than that obtained in NA. For the *P. fluorescens* strain, growth was void at the high concentrations ranging from C_1=4% to C_4=0.5%. From C_5=0.25%, the growth became noticeable, then reached a maximum of 165.3 colonies/dish at C_7=0.06%. Optimal efficiency was obtained at concentration C_7 = 0.06%, where the growth was maximum (165.3 colonies/dish). At this concentration, which represents a RC of only 3% of the NA medium, a relative growth RG=244.17% was obtained.

Effect of starved earthworm extract (FE) on bacterial growth

The effect of the crude FE extract at different C_n concentrations (C_1, ..., C_8) on the growth of *E. coli* and *P. fluorescens* gave the results presented in

Table 2. Growth of *E. coli* and *P. fluorescens* in solid media based on crude extract of freshly harvested earthworms (FHE) at different concentrations (C_n: g/100ml).

			Solid media based on crude extract earthworm (FHE) at different concentrations (C_n)								NA[e]
			C_1=4%	C_2=2%	C_3=1%	C_4=0.5%	C_5=0.25%	C_6=0.12%	C_7=0,06%	C_8=0.03%	
E. coli	NCF[a]		30±1.15[a]	35±2.8[ab]	181±1.52[f]	77.33±1.76[e]	74.33±3.71[e]	67.333±1.2[de]	54.66±2.9[c]	42±3.05[b]	56.33±2.02[cd]
	$E_{FHE/NA}$[b]	RC[c] (%)	200	100	50	25	12.5	6	3	1.5	100
		RG[d] (%)	53.26	62.13	321.32	137.28	131.95	119.53	97.04	74.56	100
P. fluorescens	NCF		0	0	0	0	35.7±2,3[a]	87.7±11.8[b]	165.3±12.7[c]	143±3.5[c]	67.7±1.2[ab]
	$E_{FHE/NA}$[b]	RC[c] (%)	-	-	-	-	12.5	6	**3**	1.5	100
		RG[d] (%)	-	-	-	-	52.73	129.54	244.17	211.23	100

[a]Average number (± standard error) of colonies formed. [b]Effectiveness of the FE extract. [c]Relative concentration. [d]Relative growth. [e]Nutrient Agar. The letters are significantly different at the confidence level of 0.05 between culture media (Degree of significance by Tukey test ($p \leq 0.005$)).

Table 3. Growth of *E. coli* and *P. fluorescens* in solid media based on crude extract of earthworms previously deprived of soil and food (FE) at different concentrations (C_n: g/100ml).

			Solid media based on crude extract of earthworm (FE) at different concentrations (Cn)								NA [e]
			C_1=4%	C_2=2%	C_3=1%	C_4=0,5%	C_5=0,25%	C_6=0,12%	C_7=0,06%	C_8=0,03%	
E. coli	NCF [a]		72,67 ±4,09^{ab}	186 ±12,4^{c}	221,33 ±9,61^{c}	101,67 ±10,92^{b}	75 ±5,5^{ab}	72,33 ±5,36^{ab}	69,67 ±2,72^{ab}	50 ±2,31^{a}	56,33 ±2,02^{a}
	$E_{FE/NA}$ [b]	RC [c] (%)	200	100	50	25	12,5	6	3	1,5	100
		RG [d] (%)	129,01	330,20	392,92	180,49	133,14	128,40	123,68	88,76	100
P. fluorescens	NCF		0	0	0	0	159,66 ±4,3^{b}	194,67 ±8,6^{c}	352,33 ±3,93^{e}	256,7 ±8,2^{d}	67,7 ±1,2^{a}
	$E_{FE/NA}$ [b]	RC [c] (%)	-	-	-	-	12,5	6	3	1,5	100
		RG [d] (%)	-	-	-	-	235,83	287,55	520,43	379,17	100

[a]Average number (± standard error) of colonies formed. [b]Effectiveness of the FE extract. [c]Relative concentration. [d]Relative growth. [e]Nutrient Agar.

For *E. coli*, the average number of colonies formed increased significantly when the concentration of the FE extract decreased, reaching a maximum growth of 221.33 colonies/dish at concentration C_3=1% (Table 3). Below this concentration, the growth gradually decreased to reach a low value of 50 colonies/dish at C_8=0.03%. The zone of efficiency covered the range of concentrations between C_2=2% to C_7=0.06%. In this range, the relative concentration compared to the NA medium (RC) went from 100% to only 3%, giving a relative growth RG greater than 100%, varying from 330.20% to 123.68%. Optimal efficiency was obtained at concentration C_3 = 1%, where the growth was maximum (221.33 colonies/dish). It was noted that only C_2, C_3 and C_4 showed significantly higher results compared to NA. For the *P. fluorescens* strain, the effect of the FE extract was almost similar to that of the FHE extract. The growth was void at the high concentrations between C_1=4% and C_4=0.5%. The growth suddenly became observable (NCF=159.66 colonies/dish) at C_5=0.25% to reach its peak (352.33 colonies/dish) at C_7=0.06%, then dropped slowly (256.7 colonies/dish) at C_8 = 0.03%. This decline did not reach values lower than those of the NA media.

The efficiency zone extended between C_5=0.25% and C_8=0.03%. Optimal efficiency was obtained at C_7=0.06% as for the FHE extract, except that the value of the relative growth was greater (RG=520.43%) with an identical value for the relative concentration (RC=3%). It should be noted that for all four concentrations (C_5, C_6, C_7 and C_8), the NCF counted was significantly higher compared to the NA medium.

Comparison between the efficiency of FHE and FE extracts

Figure 1 shows the comparison of the maximum intensities of growth recorded at the optimal concentrations C_3 for FHE, C_7 for FE, and C_{NA} for conventional medium. This figure clearly shows that the growth of the two bacterial strains was in all cases (FHE, FE) and in all optimal concentrations (C_3, C_7) much greater than that obtained in the NA medium. Furthermore, the FE extract gave growth values always higher than those of the FHE extract. It was also noted that while *E. coli* supported relatively high concentrations because it grew well at C_3=1%, the *P. fluorescens* strain grew better at low concentrations (C_7=0.06%).

The efficiency of FHE and FE extracts at the optimal concentrations C_3 and C_7 is illustrated in Fig. 2. It can be seen that whatever the nature of the extract (FHE or FE) and its optimal concentration (C_3 or C_7), the RG value was always greater than 100%, signifying that the growth was greater than that of the NA media. Also, the RC value was always less than 100%, meaning that the concentration was lower than the NA medium. For the *E. coli* strain,

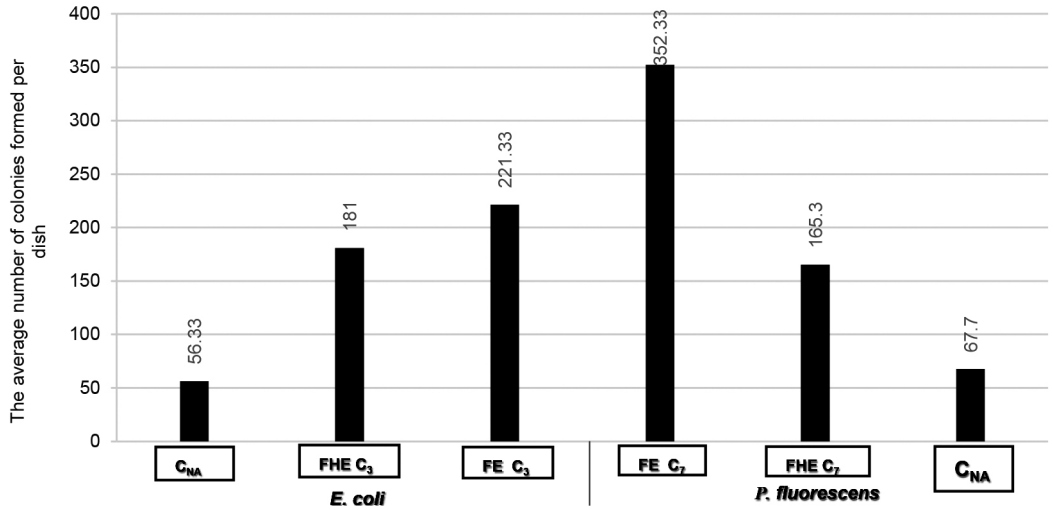

Fig. 1. Growth intensities of *E. coli* and *P. fluorescens* in conventional NA medium (C_{NA} = 2%) and in media based on extracts of FHE and FE earthworms at the same optimal concentrations (C_3 = 1% and C_7 = 0.06%). The growth rate (NCF) is expressed by the average number of colonies formed per dish.

the maximum growth was obtained in the two types of extracts at the optimal concentration C_3=1% corresponding to a relative concentration RC=50% (half less than NA). At this concentration, the relative growth in FHE extract was RG=321.32% (3.21 times greater than NA) while in the FE extract the RG was equal to 392.12% (3.92 times greater than NA). For the *P. fluorescens* strain, the maximum growth was recorded at the optimal concentration C_7=0.06% of the extracts FHE and FE. This concentration corresponds to a relative concentration RC=3% only (33.33 times lower than NA). The relative growth in the FHE extract was RG=244.17% (2.44 times greater than NA) while in the FE extract the RG was equal to 520.43% (5.20 times greater than NA).

Discussion

In this study, it was affirmed that the crude extracts of freshly harvested earthworms (FHE) and those previously deprived of soil and food for 10 days (FE) have considerable potential for bacterial growth at much lower concentrations compared to the conventional NA medium. The media based on FHE and FE earthworm extracts would therefore be more diversified in nutrients and growth substances. At extreme, low or high concentrations of the FHE and FE extracts, the intensity of growth decreased or failed depending on the bacterial strain and the nature of the extract. If at low concentrations the decrease in growth can be explained by the impoverishment of the media as a result of the dilutions effect, the decline or inhibition of growth at high

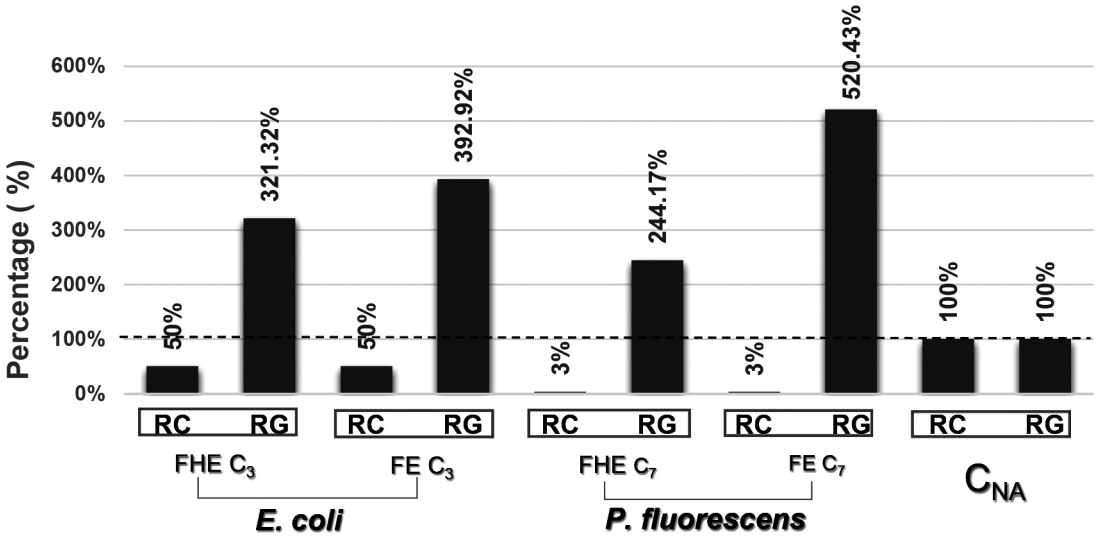

Fig. 2. Comparison of the efficiency of crude extracts of freshly harvested earthworms from (FHE) and earthworms previously deprived of soil and food (FE) on the growth of *E. coli* and *P. fluorescens*, in solid media.

concentrations could be explained by the excess of nutrients, particularly vitamins and growth factors, which are known to have toxic effects at high doses (Overmann, 2006; Zaidi and Imam, 2008).

The contents of the digestive tract of earthworms were practically not involved in improving bacterial growth. In fact, if this were the case, the efficiency of the FHE extract (full digestive tract) would be greater than that of the FE extract (empty digestive tract). The content of the digestive tract was evaluated at 6 g per 25 g dry weight of earthworms (i.e., 24%). This significant quantity consisting mainly of detritus from denser soil mixed with organic matter would not significantly contribute to the enrichment of the FHE extract. Nutrients and growth factors are therefore more endogenous to the earthworm. It was noted that in practically all concentrations, the efficiency of FE was always higher than that of FHE. Therefore, the FE extract would be qualitatively richer than FHE extract. This unexpected result could be explained by the impact of the fasting period on the physiology of the worms. Indeed, the observation of earthworms deprived of soil and food for 10 days showed that they did not return to lethargy owing to the relative humidity in the captivity pots. The activity of earthworms certainly decreased but they remained animated by movements and continued to eject casts by dejection. Thus, in order to survive, earthworms would be forced to draw on their reserves to maintain their metabolism active. The mobilization of reserves stored in the form of large insoluble molecules into small more soluble molecules which are recoverable by maceration in water and easily assimilated by bacteria would explain the richness and additional diversity of the crude extract FE.

The chemical composition of earthworms has been studied by several researchers. The protein content of earthworms varies from 32.6% to 67.2% depending on the species (Damayanti *et al*., 2008). Ghatnekar *et al*. (1995) reported that the dry matter of earthworms is 7–10% fat, 8–20% carbohydrates, 2-3% minerals, and various vitamins. In addition, earthworms increase the availability of assimilable mineral elements for bacteria and plants (Mg (5%), Ca (4%), Fe (6%) Mn (12%), Cu (6%)) (Devliegher and Verstraete, 1997). Also, they are able to produce plant growth-promoting substances in the form of rhizogenic indole compounds (El Harti and Raouane, 2009).

The results of this study, carried out entirely in the laboratory to avoid all possible interference with the chemical and biological components of the soil, can be transposed into the natural environment in order to demonstrate the post-mortem effect of earthworms at the rhizosphere level. In fact, all species of earthworms represent a biomass of 0.5 to 5 tonnes per hectare depending on the type of soil and climate (Lavelle, 1988). At the end of their life cycle, this considerable biomass breaks down releasing substances with high nutritional value for the growth and activity of bacterial communities.

P. fluorescens is of particular interest to our laboratory because it is qualified as PGPR and it is essential for soil fertility. PGPRs, in particular *Pseudomonas*, appear to be promising environmentally friendly and sustainable tools in agriculture. It has been proven that *P. fluorescens* can considerably improve plant yields (Kong *et al*. 2017). Therefore, nutrient inputs resulting from the decomposition of earthworm cadavers would constitute an alternative biological amendment, particularly in soils initially poor or impoverished by modern agricultural practices. It should be noted that the water content in *A. molleri* was evaluated in this study at 75%. If this content is expressed in relation to the biomass of the earthworms, a water mass varying from 0.37 to 3.75 tonnes per hectare will be obtained. This significant amount of water could contribute to maintaining relative humidity in the rhizosphere.

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