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Earthworms (*Amyntas* spp.) increase common bean growth, microbial biomass, and soil respiration

Minhocas (*Amyntas* spp.) aumentam o crescimento do feijoeiro, a biomassa microbiana e a respiração do solo

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Abstract

Few studies have evaluated the effect of earthworms on plants and biological soil attributes, especially among legumes. The objective of this study was to evaluate the influence of earthworms (*Amyntas* spp.) on growth in the common bean (*Phaseolus vulgaris* L.) and on soil biological attributes. The experiment was conducted in a greenhouse using a completely randomized design with five treatments and eight repetitions. The treatments consisted of inoculation with five different quantities of earthworms of the genus *Amyntas* (0, 2, 4, 6, and 8 worms per pot). Each experimental unit consisted of a plastic pot containing 4 kg of soil and two common bean plants. The experiment was harvested 38 days after seedling emergence. Dry matter and plant height, soil respiration, microbial respiration, microbial biomass, and metabolic quotient were determined. Earthworm recovery in our study was high in number and mass, with all values above 91.6% and 89.1%, respectively. In addition, earthworm fresh biomass decreased only in the treatment that included eight earthworms per pot. The presence of earthworms increased the plant growth and improved soil biological properties, suggesting that agricultural practices that favor the presence of these organisms can be used to increase the production of common bean, and the increased soil CO₂ emission caused by the earthworms can be partially offset by the addition of common bean crop residues to the soil.

Key words: Carbon. Legumes. Macrofauna. Soil ecology.

Resumo

Poucos estudos têm avaliado o efeito de minhocas nas plantas e nos atributos biológicos do solo, principalmente em plantas leguminosas, como o feijoeiro (*Phaseolus vulgaris* L.). Assim, o objetivo deste trabalho foi avaliar a influência de minhocas (*Amyntas* spp.) no crescimento do feijoeiro e nos atributos biológicos do solo. O experimento foi realizado em casa de vegetação em delineamento completamente casualizado, com cinco tratamentos e oito repetições. Os tratamentos foram constituídos de cinco níveis de inoculação (0, 2, 4, 6 e 8 minhocas por vaso) de minhocas do gênero *Amyntas* spp. Cada unidade experimental foi composta por um vaso de plástico, contendo 4 kg de solo e duas plantas de feijoeiro. O período experimental foi encerrado após 38 dias da emergência das plantas. Foram determinadas a matéria seca e a altura das plantas, a respiração edáfica, a respiração microbiana, a biomassa microbiana do solo e o quociente metabólico. A recuperação de minhocas foi alta em número e massa, com todos os valores acima de 91,6% e 89,1%, respectivamente. Adicionalmente, a massa de minhocas diminuiu apenas no tratamento com oito minhocas por vaso. A presença de minhocas

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aumentou o crescimento da planta e atributos biológicos do solo, sugerindo que práticas agrícolas que favoreçam a presença de minhocas podem vir a ser utilizadas para aumentar a produção de feijoeiro e, a elevação da emissão de CO₂ causada pelas minhocas pode ser parcialmente mitigada pela adição de resíduos culturais de feijoeiro no solo.

Palavras-chave: Carbono. Ecologia do solo. Leguminosas. Macrofauna.

Introduction

Nutrient cycling is influenced by a variety of soil organisms, ranging from macrofauna – organisms more than 2 mm in diameter that are responsible for the fragmentation of organic particles – to microscopic organisms that contribute directly to the mineralization process (GROENIGEN et al., 2015; SCHRÖDER et al., 2016). The microscopic organisms comprise the microbial biomass, and that biomass is influenced by any change in the soil environment, from abiotic factors (such as humidity and temperature) to biotic factors (such as the composition of the soil fauna).

The most representative organisms among the macrofauna are earthworms, as they comprise the majority of the total biomass found in soil (LEMTIRI et al., 2014). Because of the major changes that they can cause in their habitat, earthworms were called system engineers by Lavelle et al. (1997). Earthworms affect the microbial population because when they ingest soil and organic materials they also ingest protozoa, fungi, and bacteria (DRAKE; HORN, 2007). Furthermore, the deposition of coprolites by these organisms generates specific areas within the soil where the bacterial and fungal populations are comparatively higher due to the high concentration of organic compounds and nutrients in the coprolites (DRAKE; HORN, 2007; LIPIEC et al., 2015).

Plant responses to earthworms are dependent on biological mechanisms that include competition among earthworms and plants for water and nutrients; increased microbial biomass and microbial activity; parasitic damage reduction; and increased nutrient absorption by the plant (GROENIGEN et al., 2014; LEMTIRI et al., 2014). Fiuza et al. (2012) found a beneficial effect of earthworms

(*Chibui bari*) in maize and soil structure alteration, although soil microbial respiration did not change. By contrast, Lipiec et al. (2016) found earthworms had a significant influence on soil microbial functional diversity, making the soil less vulnerable to degradation and thus increasing the stability of ecologically relevant processes.

Several studies have been conducted to assess the impact of earthworms on plant growth (BERTRAND et al., 2015; BRAGA et al., 2016; MAKOTO et al., 2016; KIM et al., 2017). In a meta-analysis of 53 studies conducted between 1910 and 2013, Groenigen et al. (2014) reported that inoculation with earthworms increased crop yield, shoot matter, and root matter by 25%, 23%, and 20%, respectively. The same study confirmed that there has been little research involving legumes, while the opposite is true for grasses. The common bean is the second most cultivated legume in Brazil. It is a plant that stands out by its cultivation in almost all Brazilian states and because of its direct use in human food (CONAB, 2016). However, despite the importance of common bean, there are few studies of the effect of earthworms on bean growth and on attributes related to soil biology. Thus, the aim of this study was to evaluate the influence of earthworms (*Amyntas* spp.) on common bean growth and on soil biological attributes.

Material and Methods

Our experiment was conducted in a plastic greenhouse (without light control and with partial temperature control) in Curitiba, Paraná State, Brazil, during a period (November to December 2013) in which the cultivation of common bean is recommended in the region. The temperature control of the greenhouse had air extractors and an air

humidifying system, and it automatically activated when the temperature rose to 28°C, remaining in operation until the temperature dropped below 28°C.

The experiment was conducted using a completely randomized design with five treatments and eight repetitions. The treatments consisted of inoculation with five levels of earthworms of the genus *Amyntas* (0, 2, 4, 6, and 8 worms per pot). Each experimental unit consisted of a plastic pot (with a volume of 6 dm³) containing 4 kg of soil and two common bean plants. The substrate was a sample of Cambisol (collected in the 0-20 cm layer) that presented the following chemical properties: pH (CaCl₂ 0.01 mol L⁻¹) 5.0; organic carbon (Walkley-Black) 36.3 g dm⁻³; P (Mehlich 1) 6.1 mg dm⁻³; and Ca²⁺ (KCl 1 mol L⁻¹), Mg²⁺ (KCl 1 mol L⁻¹), K⁺ (Mehlich 1), and H+Al³⁺ at, respectively, 6.0, 3.9, 0.43, and 7.2 cmol_c dm⁻³. Before cultivation, the soil was fertilized with 200 mg P kg⁻¹ (Na₂HPO₄) and 150 mg K kg⁻¹ (KCl). We did not apply any soil liming.

The earthworms were collected using manual excavation and scavenging techniques (LEE, 1985) in an area of natural vegetation that received the addition of organic waste. The earthworms were selected according to the presence of a clitellum and were identified to the genus level by their external morphology according to Sims and Gerard (1985).

To determine the fresh weight of the earthworms, the animals were washed with deionized water, dried with paper towels, and then weighed on a centesimal precision scale. The earthworms were inoculated on the soil surface, which was previously moistened with deionized water. This procedure occurred under incident solar radiation to accelerate the penetration of the animals into the soil due to photophobia (EDWARDS; BOHLEN, 1996). During the acclimation period (seven days) the pots remained covered with a screen of non-woven fabric (1 mm mesh) to promote acclimation of the earthworms, prevent escape, identify deaths, and ensure replacement when necessary.

Five seeds of common bean (*Phaseolus vulgaris* L., cultivar IPR – Tuiuiu) inoculated with a peat inoculant containing *Rhizobium tropici* (SEMIA 4077 at a concentration of 2.0×10^9 cells g⁻¹) were sown, and seven days after seedling emergence (DAE) thinning was performed, resulting in only two plants per pot, which we watered regularly with deionized water.

Twenty-seven days after seedling emergence, CO₂ collectors were installed to determine soil respiration, according to Grisi (1995). Each pot received a kit composed of a wooden toothpick (15 cm in length and 4 mm diameter), two plastic 50 mL cups (PC), and a 300 mL acrylic chamber cover (ACC). The support was inserted into the soil to a depth of 2.0 cm. One PC was fixed to the support with wire, the other PC, containing 10 mL of NaOH (0.5 N) and replaced at every reading at 48 h intervals, was inserted into the first cup. The kit was covered by the ACC and inserted into the soil 1.0 cm deep, to capture soil CO₂. A blank test was installed under the same conditions and maintained in the same environment, but the ACC and the PC containing NaOH were surrounded by an insulating plastic film. In total, there were five solution replacements (every 48 h) over a period of 240 h. Soil respiration was estimated by titration of the samples with HCl (0.5 N) in the presence of BaCl₂ (50%) and phenolphthalein (0.1%), calculated according to Anderson (1982).

One day after the last soil respiration determination (38 DAE), we determined the height of the plants from the soil surface to the apex of the primary meristem. Shoots were then cut about 1.0 cm from the soil surface and subjected to oven drying at 65°C for 72 h to determine dry matter production. For the removal of roots and earthworms, the soil was moistened to facilitate breakdown. The root fraction (roots + nodules) was separated, washed with tap water, and subjected to oven drying at 65°C for 72 h to determine dry matter production. The earthworms were washed with deionized water and their number and remaining biomass was

determined. Additionally, in each experimental unit a 400 g soil sample was collected, packed in a plastic bag, and kept in a BOD (Biochemical Oxygen Demand) incubator at 4°C.

Before the microbiological testing, the samples were acclimated at room temperature for 24 h. Soil microbial respiration was determined according to Alef (1995) in a modified static system using a 1 L plastic container (PCo). Dry soil (50 g from each sample) was added and the humidity was adjusted to 40% of soil water holding capacity. The PCo received a test tube containing 10 mL of deionized water (in order to maintain internal humidity and prevent sample drying) and a 50 mL plastic cup containing 10 mL of NaOH (0.5 N). The kit was incubated at 25°C for 168 h and the remaining NaOH was titrated as described for soil respiration. Soil microbial respiration was estimated according to Stotzky (1965).

Microbial biomass carbon was determined by the substrate-induced respiration method, as described by Anderson and Domsch (1980). For each sample, 50 g of dry soil was weighed and transferred to a PCo (1 L). An aqueous solution was then added, representing the amount of water required to raise the humidity to 40% of the soil water holding capacity, plus 60 mg of glucose. The PCo was tightly closed and the samples were pre-incubated for two hours in an oven at 22°C. Afterwards, the PCo received a PC containing 10 mL of NaOH (0.5 N) and was then incubated for four hours in an oven at 22°C. At the end of the incubation period the samples were titrated as described for soil microbial respiration, and the microbial biomass carbon was estimated according to Höper (2006). The metabolic quotient (qCO_2) was determined by the soil microbial respiration/microbial biomass carbon ratio, according to Anderson and Domsch (1980).

The results were subjected to the Grubbs test for determination of extreme values and the Shapiro-Wilk test to verify data normality. We performed analysis of variance (ANOVA) and Tukey tests

between the start and end numbers and the biomass of the earthworms added to the soil to see if changes occurred in the earthworms during the experiment. The other data were analyzed by ANOVA, regression analysis, and Pearson correlation. For quadratic models the maximum point of Y (dependent variable) as a function of X (number of earthworms per pot) was obtained by equating the first derivative of each regression equation to zero.

Results and Discussion

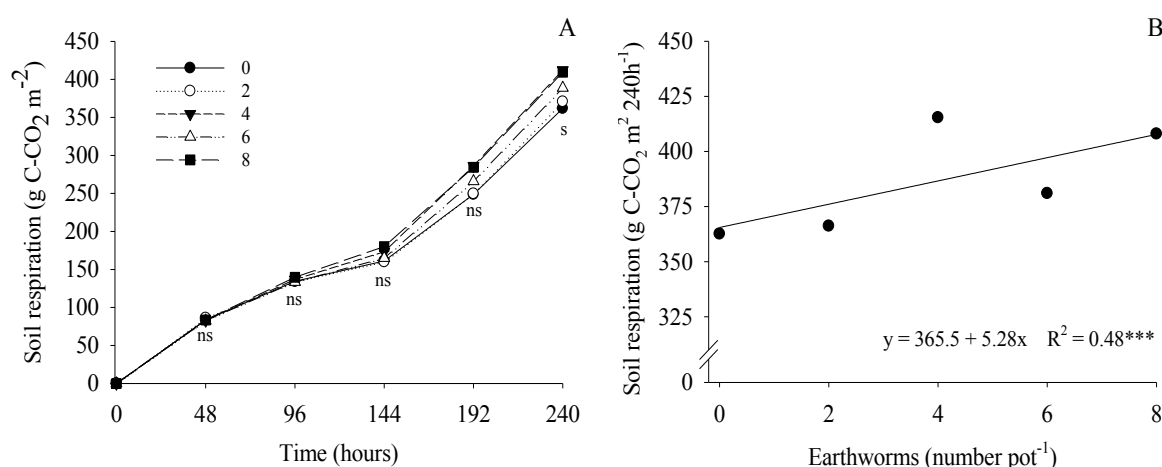
Earthworm recovery in our study was high in number and mass, with all values above 91.6% and 89.1%, respectively (Table 1). These high recovery rates are noteworthy because as Groenigen et al. (2014) reported in a meta-analysis, earthworm survival rates above 50% tend to have a greater beneficial effect on plants. In our experiment, earthworm fresh biomass decreased only in the treatment that included eight earthworms per pot (Table 1). These results suggest the need for supplementation with organic matter in future studies to ensure better environmental conditions for the earthworms when their density is high. To get around this, Bertrand et al. (2015) successfully added, weekly, 0.80 g of dehydrated alfalfa on the soil surface in pots containing two *Lumbricus terrestris*.

With respect to the five soil respiration evaluations (at 48 h intervals), the treatments with earthworms presented significant differences only when considering the soil respiration accumulated over five evaluation periods (240 h) (Figure 1A). For that total value, soil respiration increased linearly with increasing numbers of earthworms added to the soil, with values ranging from 2.5% to 14% higher than the control (without earthworms) (Figure 1B). These results corroborate the studies of Auerswald et al. (1996) and Wessells et al. (1997), which indicate, in general, that higher earthworm density leads to higher soil respiration.

Table 1. Earthworm (*Amyntas* spp.) number, fresh biomass, and recovery at the beginning and the end of the experiment, in pots with common bean (averages of six replicates).

Number (earthworms pot ⁻¹)			Fresh biomass (g pot ⁻¹)		
Start	End	Recovery %	Start	End	Recovery %
2	2 ns	100	3.5 a	3.4 a	97.1
4	4	100	6.2 a	5.8 a	93.5
6	5.5	91.6	9.4 a	8.7 a	92.5
8	8	100	11.9 a	10.6 b	89.1

ns: not significant. Different lowercase letters in the same line indicate different means by the Tukey test ($p < 0.05$).

Figure 1. Accumulated soil respiration over time (A), and accumulated soil respiration at 240 hours in relation to the number of earthworms (*Amyntas* spp.) in the soil (B), cultivated with common bean (averages of six replicates). ns: not significant. s: significant. ***: significant 1%.

Microbial biomass carbon (Figure 2A) increased from 10.19% to 15.42% in the treatments with earthworms compared to the control (without earthworms). In relation to the increase in the number of earthworms, the microbial biomass carbon results follow a quadratic model, with 4.98 earthworms pot⁻¹ being the level that presented the highest microbial biomass carbon values; after this value there is a slight decrease in microbial biomass carbon. On the other hand, no changes were registered in soil microbial respiration and soil metabolic quotient (Figure 2B; 2C).

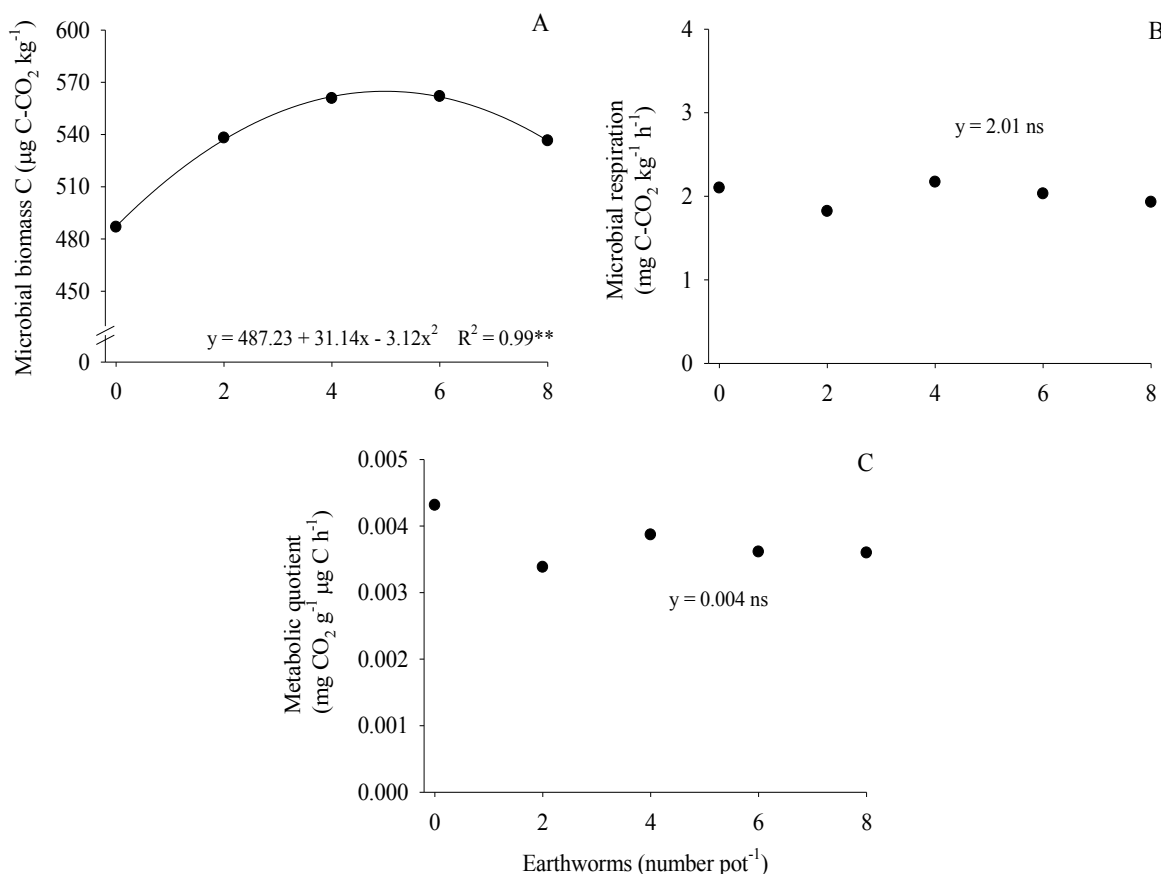
The positive influence of earthworms on soil microbial biomass has also been reported in other

studies (PASHANASI et al., 1992; AIRA et al., 2007; GROFFMAN et al., 2015), demonstrating that these animals can promote soil microorganism activity. To feed, earthworms select materials rich in the organic matter (plant-derived polymers, protozoa, fungi, and bacteria) that is their energy and nutrient source, and many microorganisms, especially bacteria, survive the passage through the digestive tract (DRAKE; HORN, 2007). Because the pH is near neutral, soluble forms of C and other compounds released in the digestive tracts of the earthworms support the proliferation of bacteria. Thus, the total number of bacteria in the digestive tract and coprolites of earthworms is generally higher than the number found in the soil in which

the earthworms live (DRAKE; HORN, 2007). Therefore, one can say that the activation of soil microorganisms by earthworms initially occurs within the earthworms themselves. Subsequently, there is an additional soil microorganism activation process due to the addition of feces rich in nutrients and organic matter (FIUZA et al., 2011; LIPIEC et al., 2015). At the same time, alterations in the soil due to the presence of earthworms can promote plant growth. Thus, higher C discharge in the rhizosphere and stimulation of the soil microbiota is expected (JONES et al., 2009). This complex of factors involving earthworm activity probably

favorable the soil microorganisms in our study, as verified by increasing microbial biomass carbon (Figure 2A). However, higher earthworm density limits microbial biomass carbon development, as seen in the decrease at higher density levels (eight earthworms pot⁻¹). This may be the result of several factors, such as the increase in soil aggregation, which prevents the use of organic carbon by soil microorganisms (MUMMEY et al., 2006). Moreover, nitrogen uptake by the plants can reduce the availability of this nutrient in the system, thereby reducing microbial biomass carbon growth (MALIK et al., 2013).

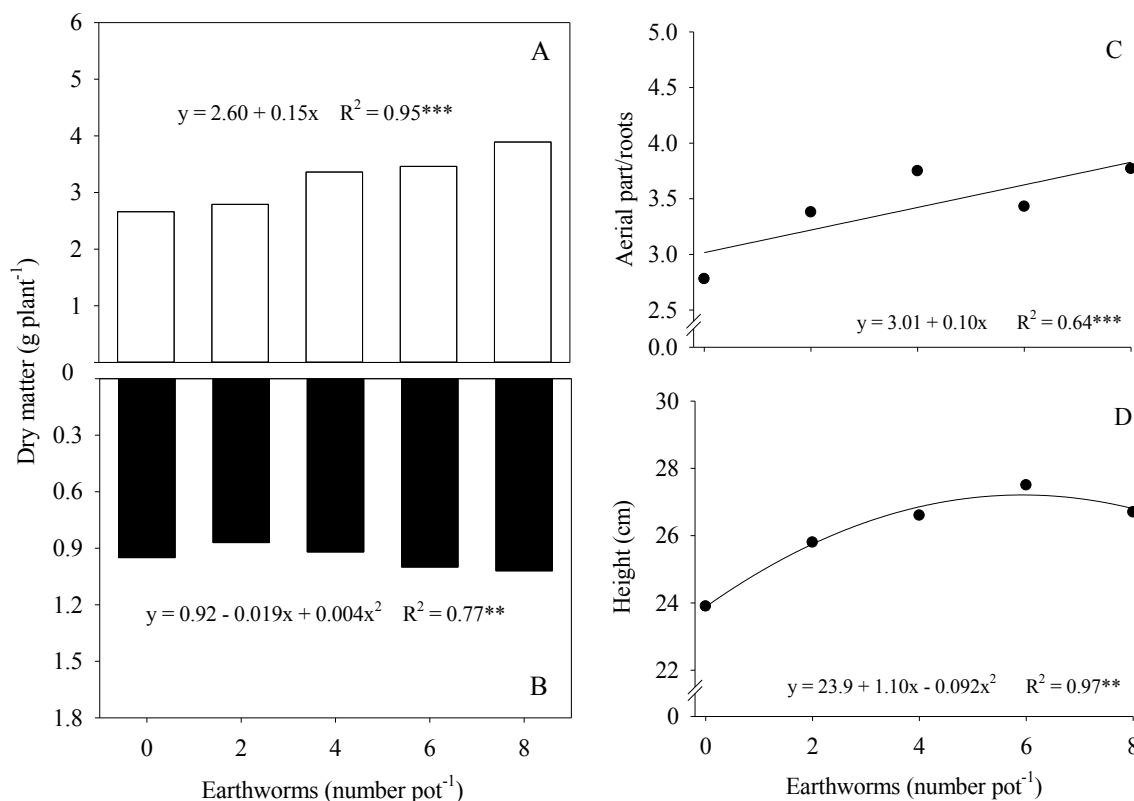
Figure 2. Microbial biomass carbon (A), microbial respiration (B), and metabolic quotient (C) in relation to the number of earthworms in the soil, cultivated with common bean (averages of six replicates). ns: not significant. **: significant 5%.



The presence of earthworms caused significant alterations in common bean growth (Figure 3). Aerial dry matter production followed a linear model, whereas the results for root dry matter

production and plant height followed a quadratic model. In addition, aerial dry matter was more positively affected than root dry matter, and thus the aerial/root ratio increased.

Figure 3. Dry matter production (DM) of the aerial part (A) and the roots (B), the aerial/root dry matter ratio (C), and the height (D) of common bean plants in relation to the number of earthworms in the soil (averages of six replicates). ** and ***, significant 5% and 1%, respectively.



The positive influence of earthworms on common bean growth that we observed (Figure 3) corroborates other studies that have evaluated the interaction between earthworms and leguminous plants (KREUZER et al., 2004; ERIKSEN-HAMEL; WHALEN, 2007). In addition, similar effects have been observed in other species, such as ryegrass (*Lolium perene* L.), barley (*Hordeum vulgare* L.), corn (*Zea mays* L.), rice (*Oryza sativa* L.), and papaya (*Carica papaya* L.) (GROENIGEN et al., 2014; XIANG et al., 2016). It is possible that nutrient availability contributed to the growth

increases, given that earthworm coprolites have a higher amount of nutrients compared to the surrounding soil (FIUZA et al., 2011; LIPIEC et al., 2015). The increase in microbial biomass carbon due to the presence of earthworms (Figure 2A) may also have resulted in an increase in nutrient availability due to nutrient cycling. Higher microbial biomass increases nutrient cycling (by means of microorganisms acting on the decomposition of organic matter), and usually also increases the activity of enzymes that have a direct effect on nutrient availability, such as phosphatase

and arylsulfatase. Although microorganisms in the short term may be a nutrient sink, they are generally considered to be a source of nutrients for plants over the long term (GRIFFITHS et al., 2012; BALOTA et al., 2014). Moreover, Puga-Freitas et al. (2012) report that earthworms can considerably affect plants by means of growth regulators present in the external medium (earthworm coprolites and/or waste released by microorganisms that are favored by the presence of earthworms), and by the activation of systemic resistance mechanisms. Under conditions in which there are serious disease problems there have also been some positive results arising from inoculation with earthworms (DIONÍSIO et al., 2014). In our study, this was

probably not significant, since neither disease nor herbivore attack were registered. At the end of the experiment, it was found that earthworms affect the soil structure through the formation of galleries. These galleries probably supported gas exchange in the soil (LEMTIRI et al., 2014), which could also have affected the growth of the bean plants. According to Brown et al. (1999), the physical changes in the soil due to the action of earthworms can also benefit plant growth.

The variables of common bean growth and the soil biological attributes showed significant correlations. In general, the correlations were positive, except for the correlation between root dry matter and soil metabolic quotient (Table 2).

Table 2. Pearson's correlations among variables of common bean plants and soil biological attributes (n = 30).

	Dry matter of aerial part	Dry matter of root	Dry matter of aerial part / dry matter of root	Soil respiration	Soil microbial respiration	Microbial biomass carbon	Metabolic quotient
Plant height	0.51***	0.11 ^{ns}	0.17 ^{ns}	0.26 ^{ns}	0.03 ^{ns}	0.16 ^{ns}	-0.29 ^{ns}
Dry matter of aerial part		0.40**	0.47***	0.53***	0.06 ^{ns}	0.23 ^{ns}	0.06 ^{ns}
Dry matter of root			-0.25 ^{ns}	0.23 ^{ns}	-0.18 ^{ns}	-0.05 ^{ns}	-0.34*
Dry matter of aerial part / dry matter of root				0.29 ^{ns}	0.24 ^{ns}	0.37**	0.26 ^{ns}
Soil respiration					0.06 ^{ns}	0.35*	0.01 ^{ns}
Soil microbial respiration						-0.24 ^{ns}	0.28 ^{ns}
Microbial biomass carbon							-0.20 ^{ns}

*, **, and ***, significant at 10%, 5%, and 1%, respectively. ns: not significant.

The positive correlations of soil respiration with aerial dry matter ($r = 0.53$) and microbial biomass carbon ($r = 0.35$) are consistent (Table 2), given that soil respiration is determined by the sum of CO₂ from soil organism respiration (added earthworms, bacteria, fungi, etc.) and root system respiration. With respect to the presence of earthworms, Lubbers et al. (2013) report that in most studies (conducted in the absence of plants) earthworms increase the emission of CO₂ from the soil in the short term (<200 days). With respect to microorganisms, even though microbial respiration (tested *in vitro*) did

not vary with earthworm presence (Figure 2B), it is possible that *in vivo* microorganism respiration, in the presence of earthworms and roots, contributed to the soil respiration increase. This could have occurred because the biomass of the microorganisms increased (Figure 2A). It is widely known that microorganisms use organic compounds such as earthworm coprolites, soil compounds, and organic compounds released into the rhizosphere, as energy sources, and as a result there is an increase in CO₂ release (JONES et al., 2009; LIPIEC et al., 2015). Finally, regarding the effect of the plants, the

correlation of soil respiration with aerial dry matter was due to increased shoot growth (Figure 3), which probably favored photosynthesis and C release into the rhizosphere. That is because an average of 12% (minimum: zero; maximum: 60%) of the C from photosynthesis is released by the roots as CO₂ into the rhizosphere (JONES et al., 2009). Moreover, the absence of correlation between soil respiration and root dry matter can be due to lower root dry matter values in the presence of two to four earthworms per pot (Figure 3).

As expected, the correlation between plant height and aerial dry matter is higher ($r = 0.51$) than that between aerial dry matter and root dry matter ($r = 0.40$). In addition, the aerial/root ratio was significantly correlated with aerial dry matter. These results occurred because the presence of earthworms has a more significant effect on the aerial part of the bean plants (Figure 3). This is probably due to the limited space in the experimental pots, and/or the abrasion of the root system by the earthworms (JANA et al., 2010). However, in a meta-analysis, Groenigen et al. (2014) reported that earthworm inoculation increases shoot matter on average by 23%, whereas the root system presented an average increase of 20%.

Although the soil metabolic quotient was not affected by the earthworms, this attribute exhibited

a slight correlation ($r = -0.34$) with root dry matter (Table 2). According to Anderson and Domsch (1980), the metabolic quotient represents the amount of CO₂ released per unit of microbial biomass C. In general, higher metabolic quotient values are found under adverse conditions, reflecting stress in the microbial community, where more oxidizable C is spent for microbial maintenance (MELLONI et al., 2001). Thus, when root dry matter decreases, the reduced deposition of labile C in the rhizosphere can create unfavorable conditions for soil microorganism development (NEERGAARDA; MAGID, 2001).

The attributes of common bean growth, soil respiration, and microbial biomass carbon showed significant positive correlations with the biomass of earthworms added to the soil (Table 3). Therefore, the correlations of initial and final added-earthworm biomass with plant and soil attributes (Table 3) followed the same trend obtained for the number of earthworms (Figures 1, 2, 3). Even with the reduction in recovered-earthworm biomass at the end of the experiment, the treatment with more added earthworms (Table 1) exhibited correlation coefficients similar to those observed for initial biomass, indicating that this reduction was not enough to alter the results.

Table 3. Pearson's correlations of fresh earthworm biomass (at the start and end of the experiment) with common bean plants and soil biological attributes (n = 30).

	Plant Height	Dry matter of aerial part	Dry matter of root	Dry matter of aerial part / dry matter of root	Soil respiration	Soil microbial respiration	Microbial biomass carbon	Metabolic quotient
Start	0.57**	0.89**	0.43*	0.51**	0.43*	-0.09 ^{ns}	0.36*	-0.24 ^{ns}
End	0.63**	0.88**	0.39*	0.50**	0.43*	-0.03 ^{ns}	0.37*	-0.28 ^{ns}

* and **, significant at 5% and 1%, respectively. ns: not significant.

Conclusion

Inoculation with earthworms increased common bean plant height, shoot dry matter, root dry matter, and shoot/root ratio.

The biological attributes of soil, respiration and microbial biomass, were favored by inoculation with earthworms.

In practical terms, agricultural practices that favor the maintenance of earthworms in the soil can be used to increase the production of common bean, and the increased soil CO₂ emission caused by the earthworms can be partially offset by the addition of common bean crop residues to the soil.

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