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Title

Short-term stabilization of grape marc through earthworms

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ABSTRACT

The winery industry generates vast amounts of organic waste during the various stages of wine production. Among the possible methodological alternatives available for its treatment, vermicomposting is one of the best-known processes for the biological stabilization of solid organic wastes by transforming them into safer and more stabilized materials suitable for application to soil. In this study we carried out a mesocosm experiment to evaluate the effectiveness of the active phase of vermicomposting for the stabilization of grape marc, an enriched lignocellulosic by-product obtained after the grape crushing and pressing stages in wine production. For this we analysed the chemical, biochemical and microbiological properties of the product resulting from this phase, in comparison with those in a control treatment. Earthworm activity reduced the abundance of both bacterial and fungal PLFA biomarkers. Decreases in microbial activity and in protease and cellulase activities were also attributed to the presence of earthworms. The differences in microbial communities were accompanied by a reduction in the labile C pool and the cellulose content. These results indicate that earthworms played a key role in the stabilization of the grape marc in the short-term, via its effects on organic matter decomposition and microbial biomass and activity.

Keywords: *Eisenia andrei*; vermicomposting; PLFA profiles; enzyme activity; microbial activity

1. Introduction

Grape marc is a lignocellulosic enriched residue that consists of the stalks, skin, pulp and seeds remaining after the grape crushing and pressing stages in wine production [1]. This by-product is a valuable resource as a soil fertilizer with high contents of macro- and micro-nutrients, primarily nitrogen and potassium for crop growth [2]. However, the overproduction of grape marc - more than 750,000 tons per year in Spain [3] - has led to inappropriate disposal practices such as the indiscriminate and inappropriately-timed application to agricultural fields. Such practices can cause serious environmental problems, including the release of excessive amounts of tannins and phenols in soils, which could inhibit root growth [4].

The environmental problems associated with the management of winery wastes could be significantly reduced by stabilizing them before their use or disposal. Stabilization involves the decomposition of an organic waste to the extent of eliminating the hazards and is normally reflected by decreases in microbial activity and concentrations of labile compounds [5]. Stabilization therefore reduces the environmental problems associated with the management of organic wastes by transforming them into safer and more stabilized materials suitable for application to soil.

Composting and vermicomposting are two of the best-known processes for the biological stabilization of solid organic wastes. Whilst composting has been widely used for the treatment of winery wastes [1,2,4,6-11], there are very few studies on the application of vermicomposting as a methodological alternative to recycling such wastes [3,12-14]. Vermicomposting involves the biooxidation and stabilization of organic material but, in contrast to composting, it depends on the joint action of earthworms and microorganisms and does not involve a thermophilic stage [15].

Microorganisms produce the enzymes that cause the biochemical decomposition of organic matter, but earthworms are crucial drivers of the process as they are involved in stimulation of microbial populations through ingestion and fragmentation of fresh organic matter, which results in a greater surface area available for microbial colonization and drastically alters biological activity [16]. Earthworms also modify microbial biomass and activity through stimulation, digestion and dispersion in casts, thereby affecting the structure and function of microbial communities [17,18]. Therefore, it is necessary to establish the effects of earthworms on the microorganisms because if the earthworms were to stimulate or depress microbiota or modify the structure and function of microbial communities, they would have different effects on the decomposition of organic matter and thus, in turn, on the stabilization of the waste.

The vermicomposting process includes two different phases with regard to the activity of earthworms: (i) an active phase during which earthworms process the waste, thereby modifying its physical state and microbial composition [17], and (ii) a maturation-like phase marked by the displacement of the earthworms towards fresher layers of undigested waste, during which the microbes take over decomposition of the waste processed by the earthworms [19]. As in composting, the duration of the active phase is not fixed and depends on the species and density of earthworms, and the rates at which they ingest and process the waste [16]. In the present study we evaluated the effectiveness of the active phase of vermicomposting for the short-term stabilization of grape marc by analysing the chemical, biochemical and microbiological properties of the product resulting from this phase.

There is some experimental evidence in the literature indicating that earthworm activity accelerates the rate of decomposition of organic matter during vermicomposting [16]. We hypothesized that this might result in a reduced microbial biomass and its

activity, and in lower enzyme activities in comparison with the control (no earthworms). We also hypothesized that these changes in microbial biomass and activity will result in a more stabilized substrate after the active phase of vermicomposting.

2. Material and methods

2.1. Substrate and experimental design

The grape marc was obtained from a vineyard in Pontevedra (Galicia, NW Spain), homogenized, stored at 5 °C until use, and turned (for aeration) and moistened with water during the two days prior to the experiment. It is a substrate rich in polysaccharides and, as such, we expected a rapid response by the earthworms and microorganisms because high amounts of easily degradable carbon compounds are available. Some chemical characteristics of the grape marc are summarized in Table 1.

The vermicomposting of grape marc was carried out in mesocosms that consisted of plastic containers (2 L), which were filled to three quarters of the capacity with moistened (80% moisture content) and mature vermicompost in order to ensure the survival of the earthworms. Five hundred juvenile and adult specimens of the epigeic earthworm species *Eisenia andrei* (220 ± 14 g fresh weight per container) were placed on the surface of the vermicompost. Specimens of *E. andrei* were collected from a stock maintained in the laboratory for one month, during which grape marc was provided as a food source. One kilogram (fresh weight) of grape marc was placed on a mesh (5 mm pore size) on the surface of the vermicompost and was rewetted by spraying it with 20 mL of tap water. The use of plastic mesh avoids mixing the grape marc and the vermicompost bedding and also facilitates the removal of grape marc after being processed by the earthworms. The mesocosms were covered with perforated lids, and placed in an incubation chamber at 20 °C and 90% relative humidity. We also included a control treatment that consisted of the grape marc incubated without earthworms.

Each treatment was replicated five times. The high density of earthworms used and the relatively rapid gut transit time of the epigeic earthworm species *E. andrei*, around 2.5–7 h, resulted in the grape marc being completely processed by the earthworms in fifteen days. After this time the samples were collected for analysis, and the biomass of earthworms was determined (233 ± 12 g fw per mesocosm).

Samples were sieved (<5 mm) in order to remove the stalks and seeds, and several parameters were determined, as detailed below.

2.2. Chemical analyses

Electrical conductivity (EC) and pH were measured in aqueous extracts (1:10, w/v). Total C and N contents were analysed in dried samples, in a Carlo Erba 1500 C/N analyser. Dissolved organic carbon (DOC) was determined colorimetrically in microplates after moist digestion ($K_2Cr_2O_7$ and H_2SO_4) of aliquots of 0.5 M K_2SO_4 extracts. Inorganic nitrogen (NH_4^+ and NO_3^-) was determined in 2N KCl extracts by acid–base titration with 0.01N HCl, in a Büchi distillation unit. Cellulose, hemicellulose and lignin contents were determined by the use of the FibreBag System (Gerhardt, Königswinter, Germany) according to the method of Goering and Van Soest [20].

2.3. Microbiological and biochemical analyses

Bacterial and fungal biomass was assessed by the phospholipid fatty acid (PLFA) analysis. The sum of Gram-positive (i15:0, a15:0, i16:0, a17:0); and Gram-negative bacteria (16:1ω7c, 17:1ω7c, cy17:0 and cy19:0) plus the marker of actinomycetes 10Me18:0 were chosen to represent the bacterial biomass; and the sum of PLFAs 18:1ω9c and 18:2ω6c was taken to indicate the fungal biomass [21]. Briefly, the total lipidic extract was obtained from 200 mg of each freeze-dried sample with 60 mL of chloroform:methanol (2:1, *v/v*), following the method described by Folch et al. [22] and modified for highly organic samples by Gómez-Brandón et al. [23]. The lipid extract

was then fractionated into neutral lipids, glycolipids and phospholipids with chloroform (5 mL), acetone (10 mL) and methanol (5 mL), on silicic acid columns (Strata SI-1 Silica (55 mm, 70A˚), 500 mg/6 mL). The fraction containing phospholipids was subjected to alkaline methanolysis [24] to obtain the fatty acid methyl esters (FAMEs), and analysed by gas chromatography-mass spectrometry (GC-MS). The detailed GC-MS experimental conditions have been described by the authors elsewhere [23]. To identify and quantify the fatty acid methyl esters, retention times and mass spectra were compared with those obtained for known standard mixtures or pure PLFAs [23].

The total microbial activity was assessed as basal respiration, by measuring the rate of evolution of CO_2, as modified by Aira et al. [18] for solid organic samples. Protease activity was measured by determining the amino acids released, after incubating the samples (1 g fresh weight) with sodium caseinate (2%) for 2 h at 50 °C, with Folin-Ciocalteu reagent, in a Microplate Reader at 700 nm [25]. Cellulase activity was estimated by determining the reducing sugars released after incubating the samples (5 g fresh weight) with carboxymethyl cellulose sodium salt (0.7%) for 24 h at 50 °C, in a Microplate Reader at 690 nm [26].

2.4. Statistical analysis

A Student's t-test was used to determine the differences between the control and the earthworm treatment. All statistical tests were evaluated at the 95% confidence level. Statistical analysis of the data was carried out with the SPSS 14.0 software programme.

3. Results and discussion

The epigeic earthworm species *E. andrei* played a key role in the stabilization of the grape marc in the short-term, via its effects on organic matter decomposition and microbial biomass and activity. The presence of this earthworm species led to a decrease in the labile C pool (DOC) of grape marc, to a greater extent than in the control

treatment (Table 2; *t*-test: P<0.05). Dissolved organic carbon generally contains organic compounds that have different susceptibilities to microbial degradation and different phytotoxic properties. For this reason the DOC composition may have an important role in determining the stabilization process [27]. As found for DOC concentration, a reduction was also observed in the content of cellulose, relative to the control, as a result of the earthworm activity (Table 2; *t*-test: P<0.05). These findings are consistent with the general hypothesis that earthworms accelerate the rate of decomposition of organic matter during vermicomposting [16,18,19,28,29]. However, there were no significant differences between samples with regard to the concentration of hemicellulose (Table 2; *t*-test: P=0.50) and lignin (Table 2; *t*-test: P=0.87) and the C to N ratio (Table 2, *t*-test: P=0.40). Namkoong et al. [30] established that this ratio could not be considered as a reliable stability index, as it changed irregularly with time. Moreover, when wastes rich in nitrogen are used as source material for vermicomposting, like sewage sludges or manures, the C to N ratio can be within the values of a stable vermicompost even though it may still be unstable.

Vermicompost stability can be also determined in terms of nitrification. Nitrogen mineralization is regulated by the availability of dissolved organic nitrogen and ammonium, the activity of the microorganisms and their relative requirements for carbon and nitrogen [31]. In our study, earthworm activity increased the concentration of NH_4^+ relative to the control (Table 2; *t*-test: P<0.01), probably because NH_4^+ is one of the excretion products of earthworms [32]; but no changes were detected in NO_3^- content (Table 2; *t*-test: P=0.60). Most of the nitrification occurs during the maturation stage, as shown by Atiyeh et al. [29] in a vermicomposting experiment with the earthworm species *E. andrei*.

Considering the key role of microorganisms in the vermicomposting process, the use of microbiological properties as stability indicators is not surprising. There is recent evidence in the literature suggesting that digestion of the organic material by these earthworm species has negative effects on microbial biomass. Indeed, Aira et al. [28] detected a decrease in microbial biomass C in casts of *Eudrilus eugeniae* fed with pig slurry. Epigeic earthworms may also affect the microbial biomass by depletion of the resources for the microbes [15]. In the present study, the activity of earthworms reduced, relative to the control, the abundance of both bacterial and fungal PLFA biomarkers after fifteen days of vermicomposting (Fig. 1; *t*-test: $P<0.0001$ and $P<0.05$, respectively). The active phase of vermicomposting also led to a reduction in the total microbial activity of grape marc, to a greater extent than in the control mesocosm (Fig. 2A; *t*-test: $P<0.0001$). This suggests that the presence of earthworms favoured the stabilization of the residue, as shown by Lazcano et al. [33]. These authors evaluated the effectiveness of the active phases of composting, vermicomposting, and a combination of composting and vermicomposting for reducing the polluting potential of cattle manure in the short-term. They found that both vermicomposting treatments produced more stabilised substrates than the active phase of composting in terms of microbial activity. Similar decreases in microbial activity were reported in short-term experiments with epigeic earthworm species [28,34]. Indeed, Aira et al. [28] observed a reduction in microbial activity in casts of *Eudrilus eugeniae* fed with pig manure, whereas in a later study, Aira and Domínguez [34] did not detect any changes in this parameter in the presence of *Eisenia fetida*. However, in the latter study, the authors observed a reduction in microbial activity when *E. fetida* was fed on cow manure rather than pig manure.

The study of enzyme activities has been shown to be a reliable tool for characterizing the state and evolution of the organic matter during vermicomposting [19,35], as they are implicated in the biological and biochemical processes that transform organic wastes into stabilised products. In addition, the measurement of enzyme activities is easy, quick and inexpensive [36], but it is difficult to establish general threshold values to apply enzyme activities as stability indexes due to the widely different organic substrates involved in the vermicomposting process. In the present study, earthworm activity greatly reduced the activities of the protease (Fig. 2B; *t*-test: $P<0.05$) and cellulase enzymes (Fig. 2C; *t*-test: $P<0.01$) in comparison with the control. These findings coincide with microbial activity data, which reinforces that a higher degree of stability was reached after the active phase of vermicomposting. Similarly, Lazcano et al. [33] reported lower values of protease activity, relative to the control, after vermicomposting and composting with subsequent vermicomposting (3 and 4.4 times lower, respectively). However, they did not find any differences in relation to this enzyme activity after the active phase of composting, indicating that the vermicomposted materials were significantly more stabilised than the compost. Aira et al. [28] also reported a reduction in the activity of protease enzyme in a short-term experiment with epigeic earthworms, but did not find any differences in cellulase activity. Aira et al. [19] observed high correlations between the microbial biomass and protease and cellulase activities, which indicate that microorganisms play an important role in shaping the patterns of these two enzymes during vermicomposting. Thus, the reduction in both enzyme activities relative to the control may be due to the lower microbial biomass as a result of earthworm activity, which probably affected enzyme production. Earthworms may also affect the activity of these enzymes by modifying the availability of C and N pools. Indeed, as stated previously, the labile C pool and the cellulose concentration

were significantly lower than in the control treatment. However, as the increase in NH_4^+ was attributed to the presence of earthworms, the reduction in protease activity may be related to the decrease in microbial biomass.

4. Conclusions

The activity of the epigeic earthworm species *E. andrei* favoured the stabilization of the grape marc after fifteen days of vermicomposting. This was reflected by the lower values of labile C pool and microbial biomass and activity in comparison with those in the control. The speed at which these transformations occurred made the active phase of vermicomposting a suitable stage for studying the relationships between earthworms and microorganisms and permitted us to understand the chemical and biological consequences of earthworm activities; which may have important implications for the development of vermicomposting as a methodological alternative for the disposal of winery wastes.

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Figure legends

Figure 1. Relative abundance (% of total) of specific PLFAs used as biomarkers of bacteria (A) and fungi (B) from the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*. Values are means ± standard error. The asterisk indicates significant differences between samples (Student's t-test).

Figure 2. Microbial activity of the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*: (A) Total microbial activity measured as basal respiration; (B) protease activity; (C) cellulase activity. Values are means ± standard error. The asterisk indicates significant differences between samples (Student's t-test).

Table 1

Chemical properties of the initial grape marc used for the experiment

pH	7.77 ± 0.01
Electrical conductivity (mS cm^{-2})	0.28 ± 0.01
Dissolved organic carbon (g g^{-1})	0.005 ± 0.0003
Cellulose (g g^{-1})	0.175 ± 0.004
Hemicellulose (g g^{-1})	0.069 ± 0.005
Lignin (g g^{-1})	0.517 ± 0.003
C to N ratio	14.37 ± 3
NH_4^+ (g g^{-1})	0.0002 ± 0.00001
NO_3^- (g g^{-1})	0.00008 ± 0.000006

Table 2

Chemical properties of the substrates obtained after incubation of grape marc for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*

	Control	*Eisenia andrei*
DOC (g g^{-1})	0.0052 ± 0.0005	0.0041 ± 0.0002[a]
Cellulose (g g^{-1})	0.169 ± 0.004	0.148 ± 0.005[a]
Hemicellulose (g g^{-1})	0.051 ± 0.008	0.040 ± 0.006
Lignin (g g^{-1})	0.531 ± 0.014	0.543 ± 0.008
C to N ratio	10.3 ± 1	9.7 ± 2
NH_4^+ (g g^{-1})	0.00013 ± 0.00001	0.00019 ± 0.00002[a]
NO_3^- (g g^{-1})	0.00008 ± 0.000006	0.00008 ± 0.000004

Values are means ± standard error.
Superscript lower case letters indicate significant differences between samples (Student's t test).

Figure 1

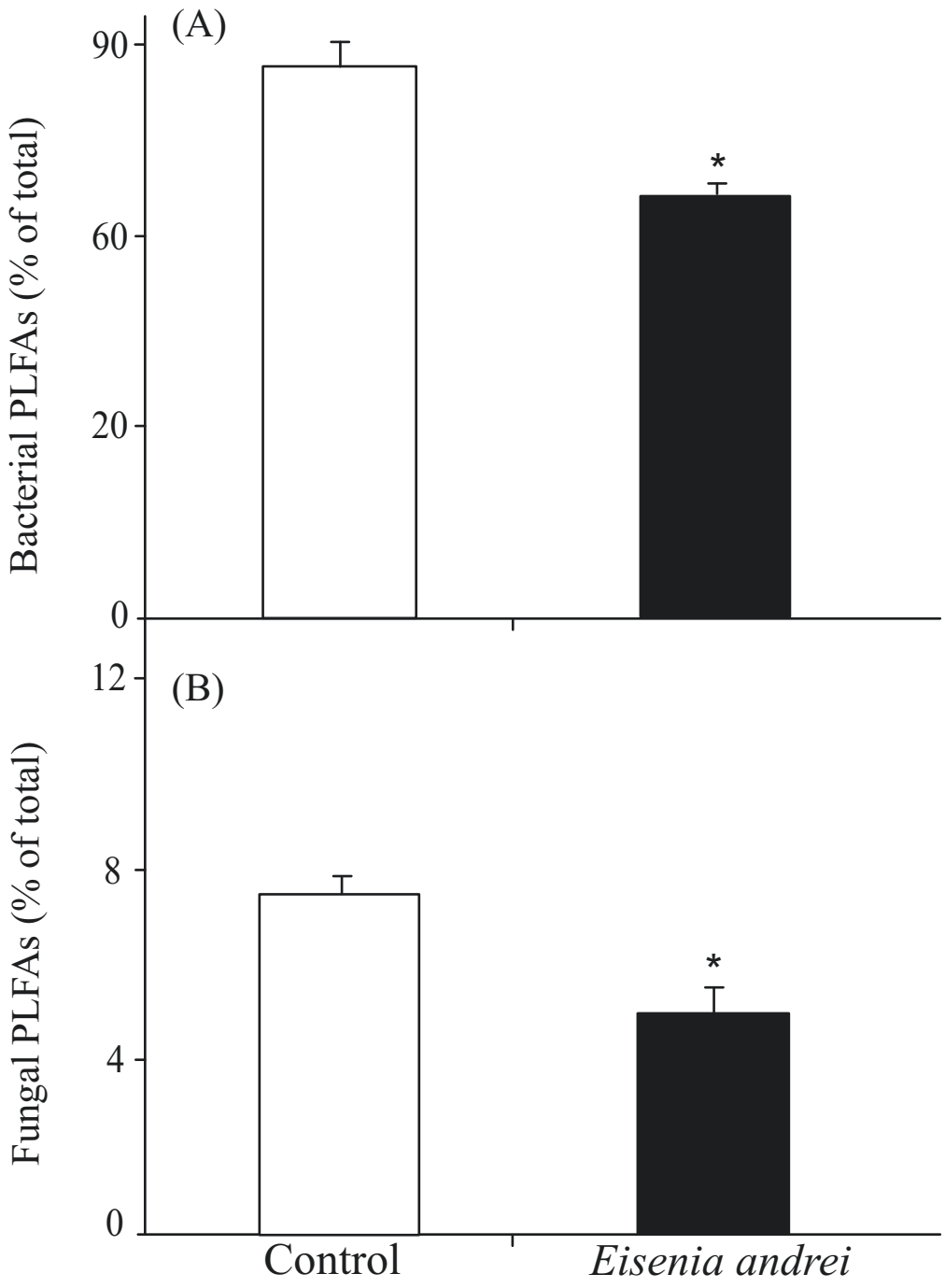

Figure 1

Figure 2

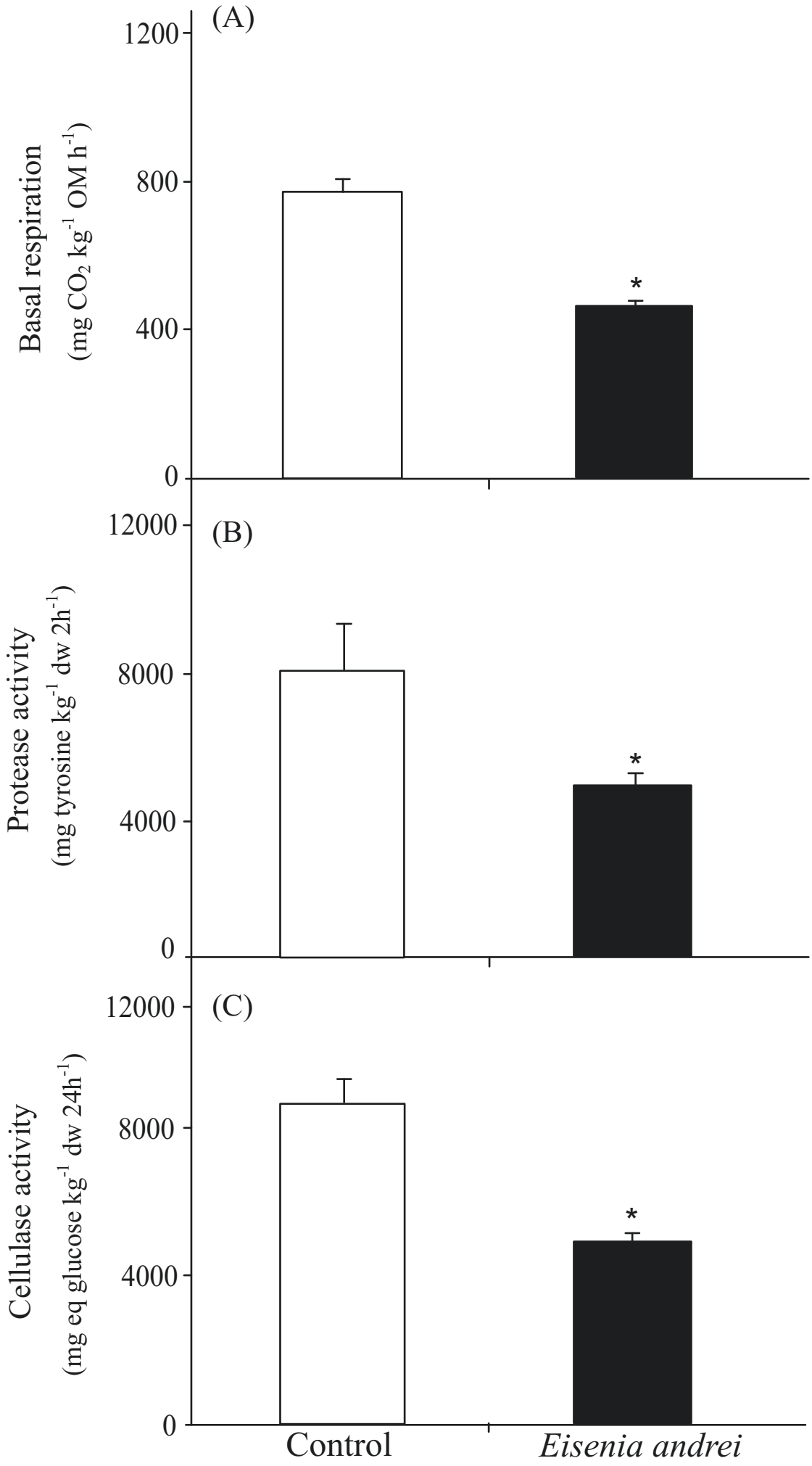

Figure 2