Soil Biology & Biochemistry 52 (2012) 13–20

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Biochar but not earthworms enhances rice growth through increased protein turnover

D. Noguera [a,b,c], S. Barot [d,*], K.-R. Laossi [e], J. Cardoso [c], P. Lavelle [a,c], M.H. Cruz de Carvalho [b]

[a] UPMC – Bioemco (UMR CNRS 7618), 32 avenue Henri Varagnat, 93143 Bondy Cedex, France
[b] Université Paris Est-Créteil – Bioemco (UMR CNRS 7618), 61 avenue du Général de Gaulle, 94010 Créteil Cedex, France
[c] Centro Internacional de Agricultura Tropical (CIAT), AA 6713, Cali, Colombia
[d] IRD – Bioemco (UMR CNRS 7618), Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Paris cedex 05, France
[e] Agronomy Department, Centre R&D Nestlé Abidjan, 01 BP 11356, Abidjan 01, Ivory Coast

ARTICLE INFO

Article history:
Received 3 December 2011
Received in revised form
26 March 2012
Accepted 5 April 2012
Available online 23 April 2012

Keywords:
Earthworms
terra preta
Biochar
Nitrogen
Protein turnover
Transcript accumulation
Proteolytic activity
Rice

ABSTRACT

The aim of this work was to compare the effects of biochar and earthworms on rice growth and to investigate the possible interactions between both. In addition to classic macroscopic variables we also monitored some leaf-level cellular processes involved in protein turnover. Both biochar and earthworms significantly increased shoot biomass production. However, biochar had a higher effect on the number of leaves (+87%) and earthworms on leaf area (+89%). Biochar also significantly increased the leaf turnover. At the cellular level, biochar but not earthworms enhanced protein catabolism by an increase in leaf proteolytic activities. This could be related to the increased expression of three of the six genes tested related to protein catabolism, one serine protease gene *OsSP2* (+24%), one aspartic acid protease gene, *Oryzasin* (+162%) and one cysteine protease gene *OsCatB* (+257%). Furthermore, biochar also enhanced the expression level of two genes linked to protein anabolism, coding for the small and large subunits of rubisco (+33% and +30%, for *rbcS* and *rbcL*, respectively), the most abundant protein in leaves. In conclusion, our data gives evidence that biochar increased rice biomass production through increased leaf protein turnover (both catabolism and anabolism) whereas earthworms also increased rice biomass production but not through changes in the rate of protein turnover. We hypothesize that earthworms increase nitrogen uptake at a low cost for the plant through a simultaneous increase in mineralization rate and root biomass, probably through the release in the soil of plant growth factors. This could allow plants to accumulate more biomass without an increase in nitrogen metabolism at the leaf level, and without having to support the consecutive energy cost that must bear plants in the biochar treatment.

1. Introduction

Many soils of the lowland humid tropics are thought to be too infertile to support sustainable agriculture. One of the major problems is the rapid decomposition of organic matter (Zech et al., 1990) due to the high temperatures, intense precipitation, and the lack of stabilizing minerals. On soils with low nutrient retention capacity the strong tropical rains easily leach available and mobile mineral nutrients limiting the efficiency of conventional fertilizers. The reduction of soil content in organic matter (SOM) is causing soil degradation. The agriculture is often not sustainable without nutrient inputs beyond 3 years of cultivation (Tiessen et al., 1994). In tropical areas, the development of techniques improving soil fertility is thus a priority. The use of more stable organic matter could help to increase the sustainability of soil fertility. In this context, biochar addition to soils is a promising alternative to transfer of more easily decomposable organic matter (Zech et al., 1990; Fearnside et al., 2001). Indeed, the existence of anthropogenic biochar-enriched dark soils (*terra preta de indio*) and the fact that they have kept a high fertility for hundreds of years supports this idea. Apart from high carbon contents, the most striking feature of biochar is its capacity to retain mineral nutrients (Glaser, 2007). The fertility of *terra preta de indio* is most likely linked to an anthropogenic accumulation of phosphorus (P), calcium (Ca), and fragmented biochar.

Another sustainable way to increase tropical soil fertility is by maintaining high biomasses of earthworms (Lavelle et al., 2001). They are known to positively affect plant growth via five main mechanisms (Scheu, 2003; Brown et al., 2004): (1) an increased mineralization of soil organic matter (2) the production of plant

* Corresponding author.
E-mail address: sebastien.barot@ird.fr (S. Barot).

0038-0717/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2012.04.004

growth substances via the stimulation of microbial activity; (3) the control of pests and parasites; (4) the stimulation of symbiotic microorganisms; (5) modifications of soil porosity and aggregation, which induces changes in water and oxygen availability to plant roots.

We have previously reported significant macroscopic effects of biochar and earthworms on rice growth in a greenhouse experiment using different soil types (Noguera et al., 2010). We have shown that (1) biochar and earthworms have additive positive effects on rice growth, (2) they differently influence resource allocation and (3) these effects depend on soil type. In the present article, we use the same greenhouse experiment but focus on the physicological effects of biochar and earthworms in the most fertile soil where differences between treatments were the most significant (Noguera et al., 2010). Ecological studies on plant responses to a particular soil treatment generally focus on root-level responses (Gregory, 2006). Very few studies have included measurements of leaf-level physiology (Day and Detling, 1990a, 1990b; Jaramillo and Detling, 1992a; Jaramillo and Detling, 1992b; Peek and Forseth, 2003; Blouin et al., 2005) and up to now the molecular processes underlying the observed changes in plant growth and morphology have seldom been addressed (Blouin et al., 2005; Jana et al., 2010; Endlweber et al., 2011). Studying the physiological and cellular processes occurring at the leaf-level in the presence of earthworms, biochar or both should therefore deepen our understanding on the mechanisms through which earthworms and biochar influence plant growth. To tackle these issues, in addition to macroscopic variables, we monitored some leaf-level cellular processes involved in leaf protein turnover.

Plant scientists have long recognized protein turnover as a fundamental component in plant development. Research has however traditionally focused on physiological processes relevant for agriculture and variety improvement, including the breakdown of storage proteins during seed germination, and protein remobilization upon the onset of leaf senescence, concomitant with the reallocation of N resources to reproductive organs (Huffaker, 1990). However, the proper functioning of a cell is ensured by the precise regulation of protein levels that in turn are regulated by a balance between the rates of protein synthesis and degradation. Therefore, we suggest that macroscopic treatments influencing plant growth should lead to different regulations of leaf protein synthesis and degradation.

Within plant cells, protein degradation is mediated by proteolysis (Callis, 1995; Schaller, 2004). Unlike other cellular enzymes, proteolytic enzymes (also termed proteases) do not have specific substrate targets and nomenclature is based on the amino acids present at the active site. There are mainly 4 super families of proteases assembled in the MEROPS database (Rawlings et al., 2008; http://merops.sanger.ac.uk/): aspartic acid proteases, serine proteases, cysteine proteases and metalloproteases. Since proteases can cleave more or less any available protein, they are present in specific cellular compartments, namely in lysosome-like acidic vacuoles (Callis, 1995; Vierstra, 1996). Protease activity is also under tight control, both at the expression and post-translational levels and also by specific inhibitors.

Nitrogen (N) is an essential macronutrient for plant growth, and crop production is often greatly affected by N nutrition. In rice seedlings, about 70% of N in the aboveground part is allocated to leaf blades and supports their photosynthetic function (Mae et al., 1984). Approximately 80% of total leaf N is invested in chloroplasts (Makino and Osmond, 1991). A number of proteins participate in photosynthetic reactions in chloroplasts, ribulose-1,5 − bisphosphate carboxylase/oxygenase (Rubisco) being the most abundant. Rubisco is both an enzyme of photosynthesis and the most abundant leaf protein. It accounts for 12–35 % of total leaf N in C_3 plants (Makino, 2003; Makino et al., 2003; Kumar et al., 2004). It comprises eight small subunits (*SSUs*) and eight large subunits (*LSUs*), which are products of the nuclear *rbcS* gene and the chloroplast *rbcL* gene, respectively. Rubisco is degraded during leaf senescence and its N is re-mobilized and translocated into growing organs and used for their growth. Rubisco-derived N is considered to account for about 40% of total re-mobilized N from senescing leaves in rice (Makino et al., 1984). Therefore, the turnover of Rubisco, namely, its synthesis and degradation, should closely be related to both C and N economy in plants (Imai et al., 2005).

In this context, our study aimed at testing the following hypotheses: First, since biochar and earthworms influence plant growth at least through an increase in mineral nutrient availability, they should influence nitrogen metabolism, namely protein turnover; Second, since biochar and earthworms influence plant growth through partially different mechanisms, they should affect plant physiology differently at the leaf cellular level. In order to test these hypotheses we measured some classic macroscopic parameters (shoot root and leaf biomasses, C/N.) and tried to relate them to the underlying processes related to leaf protein turnover operating at the cellular and molecular levels.

2. Materials and methods

2.1. Microcosms preparation

The soil was collected from a coffee plantation at Pescador, located in the Andean hillsides of the Cauca Department, southwestern Colombia (2° 48′ N 76° 33′ W). As previously described (Noguera et al., 2010) the Pescador soil is a moderately acid (pH = 5.1) Inceptisol (USDA, 1998) relatively rich in organic matter (11.5%), mineral nitrogen (12.9 mg NH_4^+–N kg^{-1}, 27 mg NO_3^-–N kg^{-1}) and with a relatively high CEC (6.0 cmol kg^{-1}). The soil was dried and sieved (2 mm mesh). Two soil treatments were implemented: soil with no addition (NB) and soil with the addition of biochar (B). Biochar has been prepared at the CIAT (Centro Internacional de Agricultura Tropical) as described previously (Rondon et al., 2007) and has been added locally around coffee plants in a long term experiment to assess biochar effect on coffee production. Our NB (B) treatment consisted in soil collected from the control (biochar) treatment of the field experiment. Taken together, the soil of our B treatment contained 25.5 g of biochar per kg of dry soil. This corresponds to 20.4 t of biochar per ha assuming that the biochar has been mixed with the first 10 cm layer of soil. Microcosm containers consisted of PVC pots of 10 cm diameter and 15 cm height. They were filled with 900 g of dry soil. The earthworm treatment consisted in the addition of five adults of *Pontoscolex corethrurus* (initial fresh weight 5 ± 0.5 g), an endogeic species common in all humid tropics (Lavelle et al., 1987).

2.2. Plant growth and experimental design

Rice plants (*Oryza sativa* cv. Linea 30) (Chatel et al., 2000) were grown in a greenhouse for three months under controlled conditions (temperature 27–29 °C, relative humidity 65–95 %, light intensity of 600 μmol m^{-2} s^{-1} and a 12-h photoperiod) as previously described (Noguera et al., 2010). Microcosms were regularly weeded during the experiment and maintained at 80% of soil field capacity (this was checked through regular weighing of the pots). Pots were arranged in a completely randomized design. Plants were submitted to four combinations of treatments: Earthworms and Biochar (EB), Biochar (B), Earthworms (E) and a Control (C) without biochar or earthworms. Five replicates were implemented per treatment.

2.3. Macroscopic measurements

The macroscopic parameters considered were the following: shoot biomass, root biomass, total number of leaves, leaf turnover (number of dead leaves/total number of leaves), total foliar area, chlorophyll concentration. At the end of the plant cycle (110 days), plant biomass (i.e. shoot and root mass) was measured after drying at 40 °C for 2 days. Leaf area and Chlorophyll concentration were measured after the harvest with a leaf area meter (LI-1300 Area meter) and a Chlorophyll Meter (Minolta SPAD 502) respectively.

2.4. Protein extraction and total proteolytic activity quantification

Leaves were collected at 65 days, frozen in liquid nitrogen and kept at −80 °C until needed. Frozen leaves (1 g) were homogenized in 450 μL 50 mM Tris–HCl buffer pH 6.8. The homogenates were transferred to Eppendorf tubes then centrifuged at 14 000 g for 20 min. The protein content of the supernatant was determined according to Bradford (1976). Proteolytic activity was assayed using bovine serum albumin (BSA, Sigma–Aldrich, France) as the substrate, under different pH. Briefly, the assay mixture contained 50 μg of the protein extract (adjusted to a final volume of 50 μL), 10 μL of 10% BSA in either 50 μL 100 mM citrate buffer for the acidic pH range (pHs 2.7, 4.2 and 5.2) or 50 mM Tris–HCl buffer for near neutral pH (pH 6.8). A blank control was made where the protein extract was replaced by 50 μL of extraction buffer (50 mM Tris–HCl, pH 6.8). The reactions were allowed to proceed for 12 h at 37 °C then stopped by the addition of 100 μL 10% trichloroacetic acid (TCA) for 1 h on ice. The mixture was then centrifuged for 15 min at 11,000 rpm. The proteolytic activity was followed by the decrease in absorbance at $\lambda = 280$ nm of the TCA soluble fraction using the blank control as zero.

2.5. Total RNA isolation

For total RNA extraction 100 mg of frozen leaf material were ground with a mortar and pestle in liquid nitrogen. The total RNA was extracted using the RNEasy Plant Minikit (Qiagen, France) according to the manufacturer's instructions. Total RNAs were quantified with a Nanodrop ND-1000 spectrophotometer (Starlab, USA) at 260 nm.

2.6. RT-PCR analysis of several genes related to protein turnover

We selected several genes involved in protein turnover in order to check if their expression patterns could be related to the cellular proteolytic activities and the macroscopic measurements. The selected genes code for known rice proteolytic enzymes (related to protein catabolism) such as two serine proteases (*OsSP1*, AB037371 and *OsSP2*, AY683198), two aspartic acid proteases (*Oryzasin*, D32144 and *OsAP1*, D12777) and two cysteine proteases (*OsCP1*, X80876 and *OsCatB*, AY916493), as well as two genes coding for the small and large subunits of rubisco (*rbcS*, D00643 and *rbcL*, L24073) (related to protein anabolism). A rice actin gene was used as a constitutive control (AF285164). Primers pairs used (Table 1) were either designed manually or using the primer3 software (Rozen and Skaletsky, 1999).

First strand cDNA synthesis was performed in 20 μL reactions on 50 ng of total RNA using 40 units of Omniscript reverse transcriptase (Qiagen, France) and 10 μM of oligo-dT primer according to the manufacturer's instructions. Transcript abundance of the genes listed above (Table 1) was analyzed by semi-quantitative RT-PCR using 5 μL of cDNA obtained from the leaves of control and treated plants and the different primer couples (15 pmol each). PCR reactions were performed in a Master Cycler Gradient thermocycler (Eppendorf AG, Germany), using the Taq PCR Master mix (Promega, France) on a 20 μL reaction volume. For each primer pair, the optimal number of cycles in order to obtain a PCR amplification outside the plateau phase was determined. PCR reactions were as follows: a first step 50 °C for 30 min, 95 °C for 15 min followed by 30–35 cycles (Table 1) (denaturation step at 95 °C for 25 s, annealing at 57–59 °C 50 s) and 7 min at 72 °C. PCR products were analyzed after separation on ethidium bromide stained 1% agarose gels. Fluorescence images of PCR products were digitized and quantified with the Gel-Doc Quantity One software (BioRad, France). Relative transcript levels were calculated with reference to the controls taken as 1.

2.7. Statistical analysis

Though the experiment has already been published (Noguera et al., 2010), all the results we present but the root and shoot biomasses of Fig. 1 are new. We display again these two variables because they are necessary to interpret the new results about the protein turnover. Statistical analyses were performed using the SAS software version 6 (SAS, 1989). ANOVAs were used to test the effect of earthworms and biochar as well has their interaction on each measured variable (using the SAS GLM procedure, sum of squares type III). To determine the direction of significant effects we used multiple comparison tests based on the least square means (hereafter LSmeans, LSmeans SAS statement), taking into account the Bonferroni correction. Residual normality and homocedasticity were verified using Kolmogorov–Smirnov and Bartlett tests. Significant differences between means are marked by different letters in the histograms.

Table 1
Primers used for the semi-quantitative RT-PCR reactions.

Gene name	Primer pair sequences (F, forward; R, reverse)	
Serine proteases		
OsSP1	F 5′ GATCACTCTGGGGGACAAGA 3′	R 5′ TTCAATGCTACCGGGAAAAG 3′
OsSP2	F 5′ TCTTCCAACTGCCAAGATCC 3′	R 5′ TGCATCAGCACTGTTCACAA 3′
Aspartic acid proteases		
Oryzasin	F 5′ CCTGATTGGAGGAAAGACCA 3′	R 5′ CACAGACCAACCTGAGAGCA 3′
OsAP1	F 5′ AAAAGTATGCAGCCAGGTTGG 3′	R 5′ TGGCAGCTGACAGTTGATTC 3′
Cysteine proteases		
OsCP1	F 5′ GGCACCAAGTACTGGATCGT 3′	R 5′ TCACAGGCTCACATCTCGTC 3′
OsCatB	F 5′ GAACCAAGTTTGGCTGGAAA 3′	R 5′ GCAAGCAGCCAGTAATCCTC 3′
Rubisco subunits		
rbcS	F 5′ GATTCGTCTACCGCGAGAAC 3′	R 5′ TTGTCGAAGCCGATGATACG 3′
rbcL	F 5′ CTTGAATGCGACTGCAGGTA 3′	R 5′ GAAGAAGTAGGCCGTTGTCG 3′
Actine	F 5′ ATCCTCCGTGGAGAAGAGCTA 3′	R 5′ GCAATGCCAGGGAACATAGT 3′

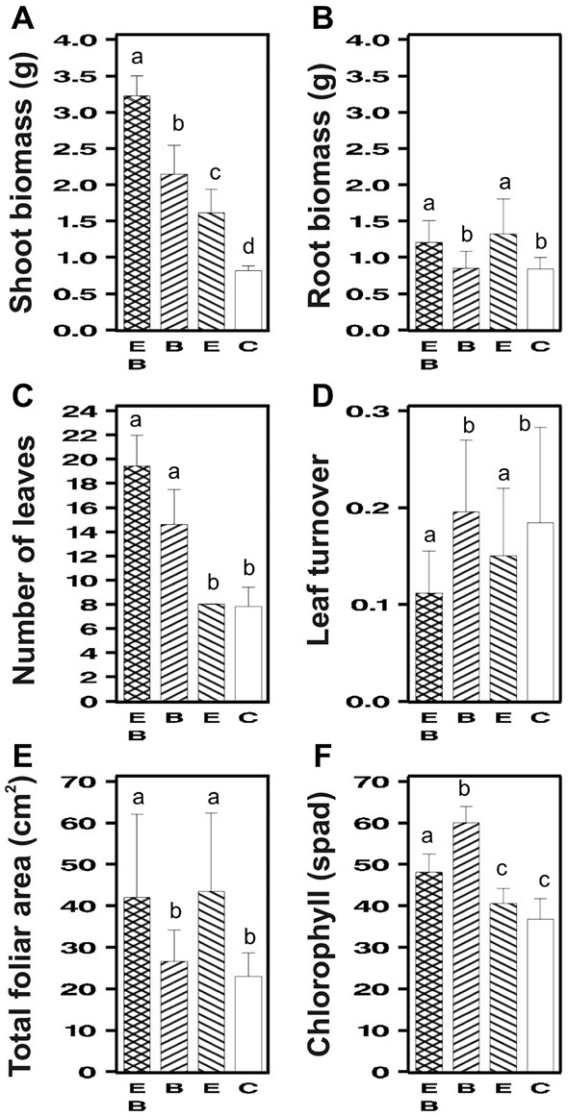

Fig. 1. Effects of four soil treatments: earthworms and biochar (EB); biochar (B); earthworms (E); and control (C) without biochar and without earthworms on *Oryza sativa* (A) shoot biomass, (B) root biomass, (C) number of leaves, (D) leaf turnover (number of dead leaves/number of leaves), (E) total foliar area and (F) chlorophyll content. Means are displayed together with standard deviations. Significant differences between means are marked by different letters (comparisons between least square means).

3. Results

3.1. Macroscopic effects of biochar and earthworms

Both biochar and earthworms increased significantly the shoot biomass (Fig. 1). However the effect of biochar was stronger than the effect of earthworms (+163% versus + 98%). Earthworms did not increase significantly the number of leaves whereas biochar did (+87% versus + 3%, Fig. 1). Although a significant interaction between biochar and earthworms was found for the number of leaves, biochar and earthworm macroscopic effects were mostly additive (Table 2, Fig. 1): i.e. earthworm effects did not change with the biochar treatment and vice versa. Root biomass followed a different pattern from the shoot biomass and the number of leaves. The strongest effect on this variable was a positive earthworm effect (+58%, Table 2, Fig. 1). Earthworms significantly decreased the leaf turnover (−37.5%) and increased the total foliar area (+89%) (Table 2, Fig. 1). Both earthworms and biochar increased the leaf chlorophyll content but a complex negative interaction between the two led to a complex pattern (Fig. 1).

Table 2
ANOVA table of *F* values for the effects of biochar (B) and earthworms (E) and their interaction (E*B) on shoot biomass, root biomass, number of leaves, leaf turnover (number of dead leaves/number of leaves), total foliar area and chlorophyll.

	df	Shoot biomass[a]	Root biomass[a]	Number of Leaves	Leaf turnover	Total foliar area	Chlorophyll
B	1	191.44***	0.19 NS	146.57***	0.42 NS	0.04	102.52***
E	1	78.31***	13.75**	11.06**	4.97*	11.71**	7.06*
E*B	1	1.82 NS	0.29 NS	9.36**	1.39 NS	0.23 NS	27.8***
R^2		0.94	0.47	0.91	0.30	0.43	0.90

Total $df = 20$.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; N.S., not significant.
[a] Adapted from Noguera et al. (2010).

3.2. Impact of biochar and earthworms on leaf proteolytic activities

The proteolytic activity of the rice leaf extracts submitted to the different treatments was studied by the capacity to hydrolyze BSA under different pHs. These pHs were used to encompass the pH optima of the different classes of proteases (Rawlings et al., 2008). The biochar treatment led to a significant increase in overall proteolytic activity, independently of the pH tested (Fig. 2). On the other hand, earthworms did not have a significant effect on leaf proteolytic activity (Fig. 2). When earthworms and biochar were used in combination, the proteolytic activities were significantly higher than the control treatment for all pHs tested and higher than the biochar treatment for the most acidic pH (2.7; Fig. 2).

3.3. Impact of biochar and earthworms on the expression of several genes related to protein turnover

RT-PCR analysis showed that the biochar treatment significantly increased the expression levels of three out of the six genes tested related to protein catabolism (proteases) (Fig. 3 a, Table 3). These include one of the serine protease genes, *OsSP2* (+24%), one of the aspartic acid protease genes, *Oryzasin* (+162%) and one of the

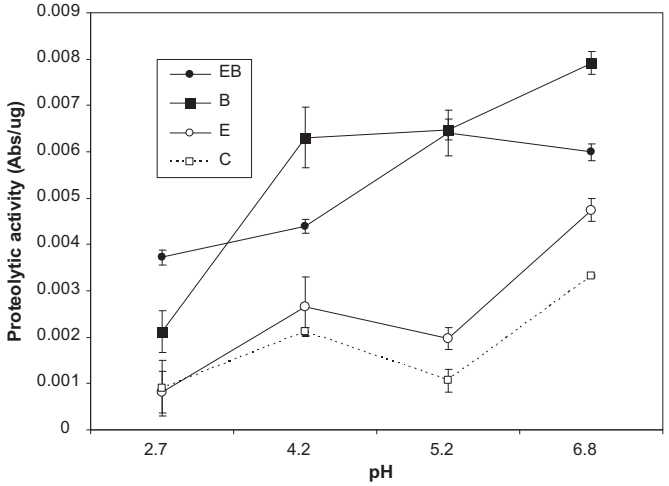

Fig. 2. Leaf proteolytic activity under different pHs in *Oryza sativa* (Linea 30) submitted to four soil treatments: earthworms and biochar (EB); biochar (B); earthworms (E) and control (C) without biochar and without earthworms. Proteolytic activity was measured using BSA as a substrate and the assay was performed as described in Materials and Methods.

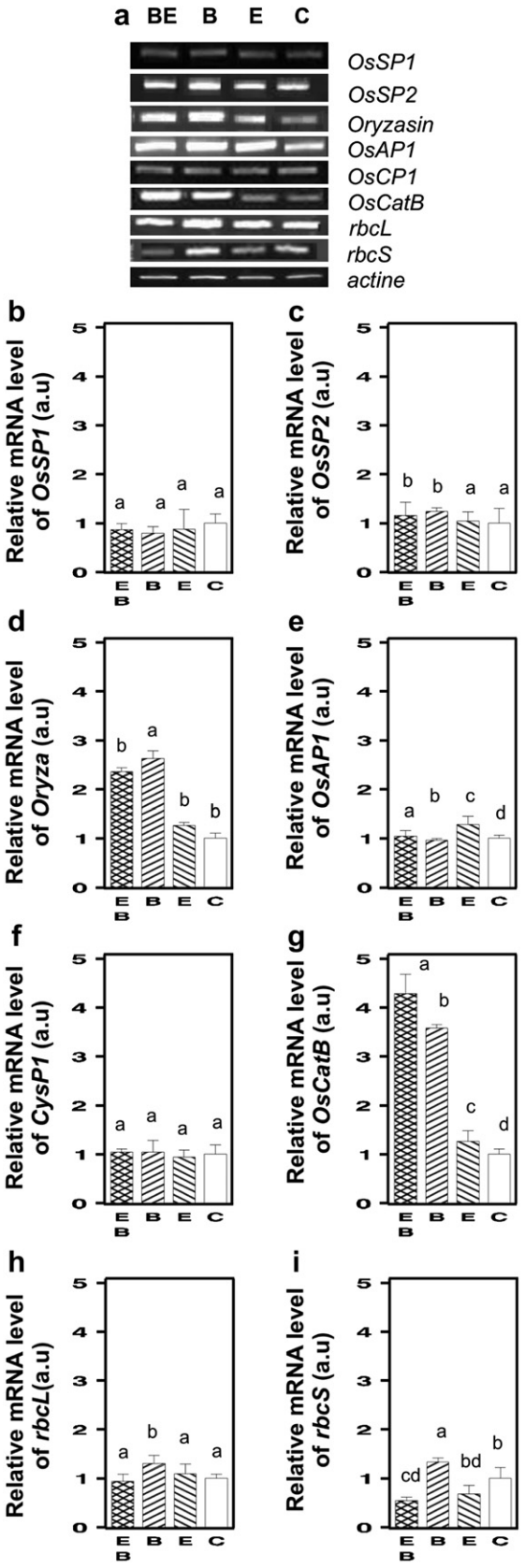

Fig. 3. Semi-quantitative gene expression of several genes related to protein turnover following RT-PCR in the leaves of *Oryza sativa* (Linea 30) under four different soil treatments: earthworms and biochar (EB); biochar (B); earthworms (E) and control (C) without biochar and without earthworms. (a) Agarose gel electrophoresis of the amplicons obtained by RT-PCR using gene specific primers of eight different proteases and actin (used as a constitutive control) in rice leaves submitted to the different soil treatments; Relative mRNA level of (b) *OsSP1*, (c) *OsSP2*, (d) *Oryzasin*, (e) *OsAP1*, (f) *CysP1*, (g) *OsCatB*, (h) *rbcL* and (i) *rbcS*. The mRNA levels were quantified with the Biorad Quantity One software. Results are representative of 3 independent assays with 3 biological replicates. Means are displayed together with standard deviations. Significant differences between means are marked by different letters (comparison between least square means).

cysteine protease genes *OsCatB* (+257%, Fig. 3, Table 3). Earthworms, on the other hand had a smaller effect on the gene expression levels of the proteases tested (Fig. 3). The only effect found was an up-regulation of the transcript level of the aspartic acid protease gene, *OsAP1* (+28%) and the cysteine protease gene *OsCatB* (+7%, Fig. 3, Table 3). When used in combination, earthworms and biochar triggered an increase in the transcript accumulation of both the aspartic acid protease genes tested, *Oryzasin* (+135%) and to a lower extent, *OsAP1* (+4%). They also significantly increased the expression levels of one of the cysteine protease genes, *OsCatB* (+328%, Fig. 3, Table 3).

Regarding the two genes related to protein anabolism (rubisco subunits), *rbcS* and *rbcL*, biochar induced an up-regulation of the expression level of both (+33% and +30%, for *rbcS* and *rbcL*, respectively, Fig. 3), but the significant interaction between biochar and earthworms (Table 3) complicated this pattern. Earthworms, on the other hand, led to a decrease in the expression level of the gene coding for the small subunit of rubisco, *rbcS* (−33%, Fig. 3, Table 3). The significant interaction between biochar and earthworms led to a decrease in the expression of *rbcS* relatively to the control (−46%, respectively, Fig. 3, Table 3).

4. Discussion

4.1. Earthworms and biochar had opposite effects on resource allocation

As far as macroscopic variables are concerned, both earthworms and biochar had a positive effect on the growth of rice and the production of shoot biomass (Fig. 1). However, when looking at the other variables more complicated patterns appear. For instance, earthworms clearly increased root biomass but not the number of leaves, whereas biochar had the opposite effect. This shows that earthworms and biochar influence biomass production but also resource allocation. While such changes in resources allocation have already been observed in the case of earthworms (Scheu, 2003; Laossi et al., 2009), they are difficult to interpret because earthworms influence plants through many mechanisms (Scheu, 2003; Brown et al., 2004) difficult to disentangle (Blouin et al., 2006). So far, the effects of biochar on resource allocation have been poorly studied (Lehmann et al., 2003; Glaser and Woods, 2004).

Our previously published results on the same experiment (Noguera et al., 2010) show that both earthworms and biochar increase the availability of mineral nutrients suggesting that this mechanism (which is the most commonly cited both for earthworms and biochar) has played an important role in our experiment and partially explains the macroscopic effects in terms of biomass. However, the fact that earthworms decreased the shoot/root ratio and that biochar had the opposite effect (Noguera et al., 2010) suggests that other mechanisms are involved and that these mechanisms differ between earthworms and biochar. The increase in root biomass in the presence of earthworms could be due to the increase in nutrient availability and would be a hint of the foraging strategies that plants have evolved to take advantage of variations in nutrient availability in space and time (Campbell et al., 1991; Scheu et al., 1999; Kreuser et al., 2004). In this case, it

Table 3
ANOVA table of *F* values for the effects of biochar (B) and earthworms (E) and their interaction (E*B) on Aspartic proteases: *OsSP1*, *OsSP2*, *Oryzasin*, *OsAP1*, *CysP1* and *OsCatB* and rubisco: *rbcL* and *rbcS*.

	df	*OsSP1*	*OsSP2*	*Oryzasin*	*OsAP1*
B	1	3.89 NS	11.96*	2744.19***	220.04***
E	1	0.29 NS	0.27 NS	0.08 NS	258.69***
E*B	1	3.23 NS	1.27 NS	104.27***	52.21***
R^2		0.480	0.62	0.997	0.985
	df	*CysP1*	*OsCatB*	*rbcL*	*rbcS*
B	1	2.80 NS	2726.46***	3.92 NS	7.56*
E	1	0.59 NS	81.94***	14.33**	249.67***
E*B	1	0.52 NS	17.66***	39.33***	42.16***
R^2		0.328	0.997	0.878	0.973

Total $df = 12$.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; N.S., not significant.

is surprising that biochar, which also increased mineral nitrogen availability (although less clearly for ammonium), did not have the same effect (Noguera et al., 2010). One explanation would be that earthworms also manipulate plant resource allocation directly through the release of plant growth factors (probably via the stimulation of bacteria) in the soil (Muscolo et al., 1999; Nardi et al., 2002; Quaggioti et al., 2004). Another experiment comparing plant response to earthworms between different fertilization treatments supports this hypothesis (Blouin et al., 2006).

4.2. Earthworms and biochar had opposite effects on protein metabolism

The earthworm and biochar effects on plant nitrogen content (Noguera et al., 2010) support the rationale above. First, both earthworms and biochar increased the total quantity of nitrogen absorbed by rice plants showing that availability of nutrient is one of the underlying mechanisms in the effects on biomass production (Noguera et al., 2010). Second, while earthworms increased the leaf N concentration (therefore decreasing the leaf C/N), as often reported, biochar had the opposite effect (Noguera et al., 2010). This again suggests that earthworms and biochar do not increase rice biomass through the same mechanisms. A possible explanation would be that earthworms both increase mineral nitrogen availability and rice capacity to uptake it (decrease in the shoot/root ratio), while biochar only increases mineral nutrient availability. Besides, the real availability for roots of cations adsorbed in the biochar particles is disputable and our results suggest that these nutrients are viewed as less available than nutrients made available by earthworm-enhanced mineralization.

What are the physiological consequences of these mechanisms in terms of protein turnover? As predicted in the introduction an effect on protein metabolism has been detected and this could be correlated to the macroscopic data. Interestingly, our second prediction is also verified: as earthworms and biochar influence rice macroscopic parameters in contrasted ways they have nearly opposite effect on protein metabolism. Biochar increased protein catabolism and to some extent protein anabolism. These effects were seen at the cellular level by the enhancement of total leaf proteolytic activity (Fig. 2) and at the molecular level by the up-regulation of the expression level of several genes related to protein catabolism and anabolism (Fig. 3). Protein turnover has been defined as the flow of amino acids from pre-existing proteins to newly formed ones (Hatfield et al., 1997). This enhancement of protein turnover can be related to the leaf dynamics under the biochar treatment. Indeed, the number of leaves produced was significantly higher for the biochar treatment than for the other treatments and the leaf turnover was higher in presence of biochar than in the presence of earthworms (Fig. 1). Taken together, this indicates a faster pace in rice development under the biochar treatment.

Acceleration of protein degradation has been related to several forms of stress (Vierstra, 1993). Furthermore, the level of protein degradation has also been directly correlated to the level of stress susceptibility (Cruz de Carvalho et al., 2001). We can therefore suggest that according to our data on proteolytic activity and protease gene expression, rice plants could be under some sort of stress in the biochar treatment. At the molecular level this increase in protein degradation could be mainly related to enhanced gene expression of *Oryzasin* and *OsCatB* (Fig. 3). These genes respectively code for an aspartic acid protease and a cysteine protease. Previous studies have shown that both these classes of proteases are expressed in different stress situations (Cruz de Carvalho et al., 2001; Harrak et al., 2001; Simoes and Faro, 2004) and during leaf senescence (Hortensteiner and Feller, 2002; Cruz de Carvalho et al., 2004). Therefore, in our present study, these genes seem to be regulated by the presence of biochar and lead to enhanced protein catabolism. When looking at the N economy in response to biochar, although rice plants had a higher total N content (+76%), they had a lower leaf N concentration (−35%) (Noguera et al., 2010). This suggests that some form of N starvation could be occurring at the leaf level. Previous works have shown that under low N inputs there is an increase of proteolytic events (Davies and Humphrey, 1978; Cooke et al., 1979).

Interestingly, the biochar treatment also enhanced the expression of the two genes coding for the small and large subunits of rubisco, *rbcS* and *rbcL* (Fig. 3). In the present study the actual amount of rubisco synthesized in response to the different treatments was not quantified but it has been previously reported that the level of *rbcS* and *rbcL* mRNAs can be correlated to the amount of rubisco synthesized before the completion of leaf expansion (Imai et al., 2005). Therefore, although enhanced proteolysis was occurring at the leaf level (rubisco being the most abundant leaf protein is also one of the main targets of leaf proteolysis), there was simultaneously some rubisco synthesis occurring that counteracted its degradation. This would help sustaining photosynthesis functioning. The increase in leaf C/N in response to biochar further supports this hypothesis. Taken together, this indicates that in presence of biochar there is an accelerated N metabolism as seen by the increased protein turnover that should therefore be involved in the enhanced biomass production observed at the macroscopic level. Therefore, the biochar effect cannot be explained by a simple N stress. Indeed, although N starvation typically leads to an increase in protein degradation (Davies and Humphrey, 1978; Cooke et al., 1979), it also leads to a slower growth rate and decreased biomass production (Humphrey and Davies, 1975).

Earthworms, on the other hand, had less significant effects on protein turnover. Furthermore, when earthworms were combined with biochar they seemed to attenuate the biochar effects on protein turnover, slowing down protein degradation (Fig. 2). Similar results were found under biotic stress (Blouin et al., 2005). Taken together, our results indicate that the physiological/cellular effects of earthworms are not occurring at the level of protein turnover but are likely to be linked to different metabolic pathways. The effect of earthworms allowed resources to be directed towards leaf production with a greater leaf area, a decreasing number of leaves and smaller shoot biomass. Indeed, the positive effect of earthworms on root biomass indicates that plant resource allocation is directed to a better utilization of nutrients. Although earthworms had no significant effect on the rate of leaf protein turnover when compared to biochar, we cannot say that they do not have any metabolic effect. In fact, earthworms are most likely to have one, since macroscopic parameters observed at the whole

plant level must have underlying physiological/metabolic process to sustain that effect. We thus hypothesize that earthworms increase N uptake at a low cost for the plant through a simultaneous increase in the mineralization rate and root biomass, probably through the release in the soil of plant growth factors (see the discussion above about our macroscopic results). This could in turn allow plants to accumulate more biomass without an increase in nitrogen metabolism at the leaf level. Further evidence is needed to support this hypothesis. The enzymatic activity and gene expression levels of the enzymes involved in N uptake and metabolism such as nitrate reductase, glutamine synthetase and glutamate synthetase should be checked at the root level. Furthermore, the root-level proteolytic events should also be checked due to the great investment of rice grown with earthworms on root biomass.

The main difference between earthworms and biochar effects on plant metabolism would only be due to the fact that earthworms lead to the indirect release of plant growth factors. Since biochar also influences soil microbial communities (Pietikäinen and Fritze, 2000) it should however be tested in the future whether biochar stimulates groups of microorganisms that also release such growth factors. A difference between biochar and earthworms is that biochar has not coevolved with plants and microorganims. It is thus conceivable that traits allowing earthworms to stimulate the release of plant growth factors have been favoured under natural selection since earthworms would benefit of better growing plants. Although such hypotheses have never been thoroughly tested (Barot et al., 2007a) they are supported by a model (Barot et al., 2007b).

5. Conclusion

In the present work we show that biochar and earthworms have positive effects on rice growth but that these effects are the result of different underlying physiological and molecular processes. Documenting trade-offs has always been a challenge in ecology. It is probably possible to meet this need using advances in plant physiology and molecular approaches as currently shown in microbiology (Novak et al., 2006). In our work, in the presence of biochar, plants renew faster their leaves to sustain photosynthesis leading to a high rate of protein degradation and synthesis. The cost is probably paid in terms of energy (to allow for the necessary increased enzymatic activities at the leaf metabolic level) and thus respiration. Both protein degradation and synthesis require respiration energy. We hypothesize that a higher protein turnover rate should have a higher respiration cost for rice plants grown with biochar added to their soil. This could be precisely measured in the future. Besides, we have recently shown that different rice cultivars respond differently to biochar and earthworms (Noguera et al., 2011). We should test in the future whether these responses are correlated with changes in protein turnover. Overall, this combined interdisciplinary approach should provide deeper insights into the characteristics that allow plant species/genotypes to react positively to earthworms and biochar and the underlying trade-offs.

Acknowledgements

We are grateful to CIAT (TSBF CIAT Institute at Cali) for providing access to the field, logistics and laboratory facilities. We would also like to thank M. Recio, J. Polania, A. Bohorquez, V. Villegas, M. Chatel, H. Mina, E. Melo, N. Asakawa, M. P. Hurtado and J. Ricaurte for their help in the establishment and evaluation of the experiment. Plant chemistry analyses were realised in Dakar, in the "Laboratoire des Moyens Analytiques of IRD" by Patricia Moulin. This study was supported by the ANR program "Jeune Chercheur 2005" through the SolEcoEvo project, (JC05-52230). We thank two anonymous reviewers for their relevant comments.

References

Barot, S., Blouin, M., Fontaine, S., Jouquet, P., Lata, J.-C., Mathieu, J., 2007a. A tale of four stories: soil ecology, theory, evolution and the publication system. PLoS ONE 2, e1248.

Barot, S., Ugolini, A., Bekkal Brikci, F., 2007b. Nutrient cycling efficiency explains the long-term effect of ecosystem engineers on primary production. Functional Ecology 21, 1–10.

Blouin, M., Zuily-Fodil, Y., Pham-Thi, A.-T., Laffray, D., Reversat, G., Pando, A., Tondoh, J., Lavelle, P., 2005. Belowground organism activities affect plant aboveground phenotype, including plant tolerance to parasites. Ecology Letters 8, 202–208.

Blouin, M., Barot, S., Lavelle, P., 2006. Earthworms (*Millsonia anomala*, Megascolecidae) do not increase rice growth through enhanced nitrogen mineralization. Soil Biology and Biochemistry 38, 2063–2068.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Brown, G.G., Edwards, C.A., Brussaard, L., 2004. How earthworms effect plant growth: burrowing into the mechanisms. In: Edwards, C. (Ed.), Earthworm Ecology. CRC Press, pp. 13–49.

Callis, J., 1995. Regulation of protein-degradation. Plant Cell 7, 845–857.

Campbell, B.D., Grime, J.P., Mackey, J.M.L., 1991. A trade-off between scale and precision in resource foraging. Oecologia 87, 532–538.

Chatel, M., Ospina, Y., Rodriguez, F., Lozano, V., 2000. Mejoramiento de los acervos genéticos. In: Project IP-4. Improved Rice Germplasm for Latin America and the Caribbean. CIAT (Centro Internacional de Agricultura Tropical), Cali, Colombia.

Cooke, R.J., Oliver, J., Davies, D.D., 1979. Stress and protein-turnover in *Lemna minor*. Plant Physiology 64, 1109–1113.

Cruz de Carvalho, M.H., d'Arcy-Lameta, A., Roy-Macauley, H., Gareil, M., El Maarouf, H., Pham-Thi, a.-T., Zuily-Fodil, Y., 2001. Aspartic protease in leaves of common bean (Paseolus vulgaris L.) and cowpea (Vigna unguiculata L. Walp): enzymatic activity, gene expression and relation to drought susceptibility. FEBS Letters 492, 242–246.

Cruz de Carvalho, M.H., Pham-Thi, A.T., Gareil, M., d'Arcy-Lameta, A., Zuily Fodil, Y., 2004. Isolation and characterisation of an aspartic proteinase gene from cowpea (*Vigna unguiculata* L. Walp.). Journal of Plant Physiology 161, 971–976.

Davies, D.D., Humphrey, T.J., 1978. Amino-acid recycling in relation to protein turnover. Plant Physiology 61, 54–58.

Day, T.A., Detling, J.K., 1990a. Changes in grass leaf water relations following bison urine deposition. American Midland Naturalist 123, 171–178.

Day, T.A., Detling, J.K., 1990b. Grassland patch dynamics and herbivore grazing preference following urine deposition. Ecology 71, 180–188.

Endlweber, K., Krome, K., Welzl, G., Schaffner, A.R., Scheu, S., 2011. Decomposer animals induce differential expression of defence and auxin-responsive genes in plants. Soil Biology and Biochemistry 43, 1130–1138.

Fearnside, P.M., Graca, P., Rodrigues, F.J.A., 2001. Burning of Amazonian rainforests: burning efficiency and charcoal formation in forest cleared for cattle pasture near Manaus, Brazil. Forest Ecology and Management 146, 115–128.

Glaser, B., 2007. Prehistorically modified soils of central Amazonia: a model for sustainable agriculture in the twenty-first century. Philosophical Transactions of the Royal Society B-Biological Sciences 362, 187–196.

Glaser, B., Woods, W., 2004. Amazonian Dark Earths: Explorations in Space and Time. Springer, New York, 213 pp.

Gregory, P., 2006. Plant Roots. Blackwell Publishing, Oxford, 318 pp.

Harrak, H., Azelmat, S., Baker, E.N., Tabaeizadeh, Z., 2001. Isolation and characterization of a gene encoding a drought-induced cysteine protease in tomato (*Lycopersicon esculentum*). Genome 44, 368–374.

Hatfield, P.M., Gosink, M.M., Carpenter, T.B., Vierstra, R.D., 1997. The ubiquitin-activating enzyme (E1) gene family in Arabidopsis thaliana. Plant Journal 11, 213–226.

Hortensteiner, S., Feller, U., 2002. Nitrogen metabolism and remobilization during senescence. Journal of Experimental Botany 53, 927–937.

Huffaker, R.C., 1990. Proteolytic activity during senescence of plants. New Phytologist 116, 199–231.

Humphrey, T.J., Davies, D.D., 1975. New method for measurement of protein turnover. Biochemical Journal 148, 119–127.

Imai, K., Suzuki, Y., Makino, A., Mae, T., 2005. Effects of nitrogen nutrition on the relationships between the levels of rbcS and rbcL mRNAs and the amount of ribulose 1 center dot 5-bisphosphate carboxylase/oxygenase synthesized in the eighth leaves of rice from emergence through senescence. Plant Cell and Environment 28, 1589–1600.

Jana, U., Barot, S., Blouin, M., Lavelle, L., Laffray, D., Repellin, A., 2010. Earthworms influence the production of above- and belowground biomass and the expression of genes involved in cell proliferation and stress responses in Arabidopsis thaliana. Soil Biology and Biochemistry 42, 244–252.

Jaramillo, V.J., Detling, J.K., 1992a. Small-scale heterogeneity in a semiarid North-American grassland .1. Tillering, N-uptake and retranslocation in simulated urine patches. Journal of Applied Ecology 29, 1–8.

Jaramillo, V.J., Detling, J.K., 1992b. Small-scale heterogeneity in a semiarid North-American grassland .2. Cattle grazing of simulated urine patches. Journal of Applied Ecology 29, 9–13.

Kreuser, K., Bonkowski, M., Langel, R., Scheu, S., 2004. Decomposer animals (Lumbricidae, Collembola) and organic matter distribution affect the performance of

Lolium perenne (Poaceae) and *Trifolium repens* (fabaceae). Soil Biology and Biochemistry 36, 2005–2011.
Kumar, P., Parry, M., Mitchell, R., Ahmad, A., Abrol, Y., 2004. Photosynthesis and nitrogen-use efficiency. In: Foyer, C., Noctor, G. (Eds.), Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism. Springer, Netherlands, pp. 23–34.
Laossi, K.-R., Noguera, D.C., Bartolomé-Lasa, A., Mathieu, J., Blouin, M., Barot, S., 2009. Effects of endogeic and anecic earthworms on the competition between four annual plants and their relative reproduction potential. Soil Biology and Biochemistry 41, 1668–1773.
Lavelle, P., Barois, I., Cruz, I., Fragoso, C., Hernandez, A., Pineda, A., Rangel, P., 1987. Adaptative strategies of *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta), a peregrine geophagous earthworm of the humid tropics. Biology and Fertility of Soils 5, 188–194.
Lavelle, P., Barros, E., Blanchart, E., Brown, G., Desjardins, T., Mariani, L., Rossi, J.-P., 2001. SOM management in the tropics: why feeding the soil macrofauna? Nutrient Cycling in Agroecosystems 61, 53–61.
Lehmann, J., Pereira da Silva, J.J., Steiner, C., Nehls, T., Zech, W., Glaser, B., 2003. Nutrient availability and leaching in an archaeological Anthrosol and a Ferralsol of the Central Amazon basin: fertilizer, manure and charcoal amendments. Plant and Soil 249, 343–357.
Mae, T., Kai, N., Makino, A., Ohira, K., 1984. Relation between ribulose bisphosphate carboxylase content and chloroplast number in naturally senescing primary leaves of wheat. Plant and Cell Physiology 25, 333–336.
Makino, A., 2003. Rubisco and nitrogen relationships in rice: leaf photosynthesis and plant growth. Soil Science and Plant Nutrition 49, 319–327.
Makino, A., Osmond, B., 1991. Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. Plant Physiology 96, 355–362.
Makino, A., Mae, T., Ohira, K., 1984. Relation between nitrogen and ribulose-1,5-bisphosphate carboxylase in rice leaves from emergence through senescence. Plant and Cell Physiology 25, 429–437.
Makino, A., Sakuma, H., Sudo, E., Mae, T., 2003. Differences between maize and rice in N-use efficiency for photosynthesis and protein allocation. Plant Cell and Physiology 44, 952–956.
Muscolo, A., Bovalo, F., Gionfriddo, F., Nardi, S., 1999. Earthworm humic matter produces auxin-like effects on *Daucus carota* cell growth and nitrate metabolism. Soil Biology and Biochemistry 31, 1303–1311.
Nardi, S., Pizzeghello, D., Muscolo, A., Vianello, A., 2002. Physiological effects of humic substances on higher plants. Soil Biology & Biochemistry 34, 1527–1536.
Noguera, D., Rondon, M., Laossi, K.-R., Hoyos, V., Lavelle, P., Cruz de Carvalho, M.H., Barot, S., 2010. Contrasted effect of biochar and earthworms on rice growth and resource allocation in different soils. Soil Biology & Biochemistry 42, 1017–1027.
Noguera, D., Laossi, K.-R., Lavelle, P., Cruz de Carvalho, M.-H., Asakawa, N., Botero, C., Barot, S., 2011. Amplifying the benefits of agroecology by using the right cultivars. Ecological Applications 21, 2349–2356.
Novak, M., Pfeiffer, T., Lenski, R.E., Sauer, U., Bonhoeffer, S., 2006. Experimental tests for an evolutionary trade-off between growth rate and yield in E-coli. American Naturalist 168, 242–251.
Peek, M.S., Forseth, I.N., 2003. Enhancement of photosynthesis and growth of an aridland perennial in response to soil nitrogen pulses generated by mule deer. Environmental and Experimental Botany 49, 169–180.
Pietikäinen, J., Fritze, H., 2000. Charcoal as a habitat for microbes and its effect on the microbial community of the underlying humus. Oikos 89, 231–242.
Quaggioti, S., Ruperti, B., Pizzeghello, D., Francioso, O., Tugnoli, V., Nardi, S., 2004. Effect of low molecular size humic substances on nitrate uptake and expression of gennes involved in nitrate transport in maize (Zea mays L.). Journal of Experimental Botany 55, 803–813.
Rawlings, N., Morton, F., Kok, C., Kong, J., Barrett, A., 2008. MEROPS: the peptidase database. Nucleic Acids Research 36, D320–D325. http://merops.sanger.ac.uk/.
Rondon, M.A., Lehmann, J., Ramirez, J., Hurtado, M., 2007. Biological nitrogen fixation by common beans (*Phaseolus vulgaris* L.) increases with bio-char additions. Biology and Fertility of Soils 43, 699–708.
Rozen, S., Skaletsky, J., 1999. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, M.S. (Ed.), Methods in Molecular Biology. Humana Press, Totowa, pp. 365–386.
SAS, 1989. SAS/STAT User's Guide, Ver. 6, fourth ed. SAS Inst., Cary, NC, 1686 pp.
Schaller, A., 2004. A cut above the rest: the regulatory function of plant proteases. Planta 220, 183–197.
Scheu, S., 2003. Effects of earthworms on plant growth: patterns and perspectives. Pedobiologia 47, 846–856.
Scheu, S., Theenhaus, A., Jones, T.H., 1999. Links between the detritivore and the herbivore system: effects of earthworms and collembola on plant growth and aphid development. Oecologia 119, 541–551.
Simoes, I., Faro, C., 2004. Structure and function of plant aspartic proteinases. European Journal of Biochemistry 271, 2067–2075.
Tiessen, H., Cuevas, E., Chacon, P., 1994. The role of soil organic matter in sustaining soil fertility. Nature 371, 783–785.
Vierstra, R.D., 1993. Protein-degradation in plants. Annual Review of Plant Physiology and Plant Molecular Biology 44, 385–410.
Vierstra, R.D., 1996. Proteolysis in plants: mechanisms and functions. Plant Molecular Biology 32, 275–302.
Zech, W., Haumaier, L., Hempfling, R., 1990. Ecological aspects of soil organic matter in tropical land use. In: McCarthy MR, B.P. (Ed.), Humic Substances in Soil and Crop Sciences: Selected Readings. American Society of Agronomy and Soil Science Society of America, Madison, pp. 187–202.