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INTERACTIONS ENTRE "*TERRA PRETA*" ET VERS DE TERRE : ANALYSE DE LEURS EFFETS SUR LA CROISSANCE ET LA PHYSIOLOGIE DES PLANTES

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Résumé

La fertilité des sols tropicaux est souvent peu durable dans les systèmes de cultures et les pâturages. Ce problème de fertilité est en grande partie du à la très rapide décomposition de la matière organique dans ces sols et à leur faible capacité à retenir les nutriments minéraux. De plus, dans un contexte où une grande partie des agriculteurs ont peu accès aux intrants (engrais minéraux) il est important de trouver des méthodes de gestion durable et nécessitant peu d'intrants. Une solution pourrait être d'enrichir le sol en charbon de bois fragmenté pour créer des "*terres noires*" comme celles découvertes récemment en Amazonie et qui auraient été implantées il y a des siècles par des groupes amérindiens aujourd'hui disparus. Une autre solution pourrait être de promouvoir des systèmes agricoles favorables aux vers de terre dont les activités augmentent généralement la croissance des plantes. Les mécanismes impliqués commencent à être connus tant pour les vers que pour les terres noires mais les interactions entre vers et terres noires n'ont jamais été abordées.

J'ai étudié dans une série d'expériences l'effet de vers de terre (*P. corethrurus*), du charbon bois et de leur interaction sur le croissance du riz: 1) réponse du riz dans différents types du sol (expérience en microcosmes), 2) réponse des 5 variétés du riz dans un type sol et dans deux niveaux de fertilisation minérale (expérience en microcosmes), 3) réponse physiologique et cellulaire du riz à la présence de vers de terre et de charbon bois et 4) effet des vers de terre sur la formation de "*terres noires*" et leur fertilité (expérience en mésocosmes).

Nous avons ainsi montré que les vers de terre et le charbon bois affectent la croissance des plantes et que les effets vers de terre et charbon bois varient en fonction du type de sol utilisé et diminuent avec la fertilisation minérale. Les résultats de la deuxième expérience montrent que les différentes variétés de riz répondent différemment à la présence de charbon bois et de vers. Enfin, il y a peu d'interactions significatives à court terme (3 mois) entre l'effet des vers et celui du charbon de bois. L'expérience en mésocosmes a de même montré que les vers de terre ont un effet limité sur la formation des terres noires et interagissent peu avec le charbon de bois, sur le moyen terme (2 ans). Au niveau physiologique, dans le sol où les vers et le charbon de bois ont un effet positif sur la croissance du riz, le charbon de bois accélère à la fois le catabolisme (protéase) et l'anabolisme (synthèse de la rubisco) de l'azote. Les vers de terre ont un effet plus modéré, avec une légère tendance à une diminution du catabolisme de l'azote.

En définitive, ma thèse fournit de nombreux éléments permettant de mieux comprendre les mécanismes d'action des vers et du charbon de bois, mais beaucoup reste à faire sur cette question. Sur le plan des applications, mes résultats montrent globalement que le charbon de bois et les vers peuvent être utilisés pour augmenter la production de riz, mais qu'il ne faut pas s'attendre à une forte synergie entre vers et charbon de bois. De plus, ils n'auront un effet positif que dans certains sols et sur certaines variétés de riz.

Mots-clés: Charbon bois, vers de terre, *Pontoscolex corethrurus*, allocation des ressources, fertilité des sols, interaction plante-sol, *terra preta nova*, métabolisme de l'azote

Abstract

The fertility of tropical soils is often poor and crop and pasture systems tend to be barely sustainable. This problem of fertility is largely due to the rapid decomposition of organic matter and the low capacity of tropical soils to retain mineral nutrients. In addition, in a context where many farmers have a very low access to mineral fertilizers, it is important to find alternative agricultural practices that are more sustainable and require less input. A solution could be to enrich soils in fragmented charcoal to create modern "*terra preta*" as those recently discovered in the Amazon and that have been implemented centuries ago by former Amerindian groups. Another solution could be to increase the biomass of earthworms in agricultural soils. Indeed, earthworms have positive effects on plant growth. While the mechanisms explaining earthworms and biochar effects on plant growth have already been studied, the effect of the combination of the two on soil fertility has never been documented.

I have thus studied, in a series of 4 experiences, the effects of earthworms (*P. corethrurus*), biochar and their interaction on the growth of rice: (1) comparison of rice responsiveness in different types of soil (microcosms experience), (2) comparison of the responsiveness of 5 rice varieties (microcosms experience), (3) physiological and molecular response of rice to biochar and earthworms, (4) earthworm effects on the maturation of "*terra preta*" (Mesocosm experience).

We have shown that earthworm and biochar affect plant growth. Earthworm and biochar effects vary with soil type and decrease with mineral fertilization. The results of the second experience show that different varieties of rice respond differently to the presence of biochar and earthworms. Finally, the short-term (3 months) effects of earthworms and biochar on plant growth are additive: there was no synergy between earthworms and biochar. The mesocosm experience has shown that earthworms have a limited effect on the formation of "*terra preta*", and that there is no synergy between biochar and earthworms on the medium term (2 years). At the physiological level, in the soil where earthworms and biochar have a positive effect on the growth of rice, biochar accelerates both the catabolism of proteins (protease) and the anabolism of proteins (rubisco synthesis). Earthworms have a moderate effect on nitrogen metabolism with a slight tendency to reduce protein catabolism.

Taken together, my thesis provides many new elements to better understand the mechanisms of biochar and earthworm actions on plant growth and soil fertility, but many questions remain totally open. In terms of applications, my results confirm that biochar and

earthworms can be used to increase rice production but that there is no synergy between the two. Additionally, biochar and earthworms would have to be used in the right soils with the right rice varieties to benefit from their action.

Key-words: Biochar, earthworms, *Pontoscolex corethrurus,* resource allocation, agroecology, soil fertility, plant-soil interaction, *terra preta nova*, nitrogen metabolism.

Sommaire

CHAPITRE 1:

INTRODUCTION GÉNERALÉ

1 Introduction générale

1.1 Fertilité des sols tropicaux

La faible fertilité des sols tropicaux constitue un des plus graves défis pour le développement rural des régions tropicales (Warner 1995). Cette fertilité est en effet affectée par une faible rétention des nutriments, les conditions environnementales du milieu, les caractéristiques physiques et chimiques des sols, et la décomposition rapide des matières organiques.

Les sols des zones tropicales ont une dominance d'argile à faible capacité d'échange cationique. Ces sols ont généralement une faible capacité de rétention des nutriments et leur fertilité se dégrade rapidement une fois la végétation naturelle remplacée par des cultures (Lehmann *et al.* 2003b). Cela limite le type de culture possible et débouche souvent sur une très faible productivité (Warner 1995). En zone tropicale humide, la chaleur et l'humidité conduisent aussi à une décomposition rapide de la matière organique des sols ce qui diminue aussi la capacité des sols à retenir les nutriments.

Un autre problème est la forte intensité des pluies, qui se traduit par des risques élevés de dégradation de la structure superficielle des sols et de l'érosion, lorsqu'ils ne sont pas protégés par une couverture végétale. Dans les régions pluvieuses, lorsque l'infiltration et le drainage profond sont importants, les risques de lixiviation des éléments nutritifs sont ainsi considérables (Jenkinson & Ayanaba 1977). En zone cultivée, ce processus est aggravé lorsque les plantes cultivées ont un enracinement peu profond ou lorsque le cycle de culture conduit à un sol nu une partie de l'année.

A la forte intensité des pluies, s'ajoute un autre problème majeur intervient dans les zones tropicales humides: la forte acidité des sols. Ces sols se caractérisent par un pH très acide (Bertrand 1983). Dans ces circonstances, l'efficacité des engrais minéraux appliquées est très faible lorsque la perte de nutriments mobiles comme NO_3^- ou K^+ est renforcée par fortes précipitations. En outre, beaucoup d'agriculteurs ne peuvent pas se permettre financièrement des applications régulières d'engrais minéraux.

Pour les raisons mentionnées si dessus, la fertilité des sols tropicaux est souvent peu durable dans les systèmes de cultures et les pâturages. Cela est particulièrement vrai quand ces systèmes sont implantés après la déforestation de parcelles forestières en Afrique ou dans

le bassin amazonien (Fearnside & Barbosa 1998; Moran 1998; Lambin *et al.* 2001). Cela est dû au fait que les sols des forêts accumulent un peu de matière organique qui disparait très vite par érosion et décomposition quand les sols sont mis à nus au moment où la forêt a été coupée (Zech *et al.* 1997). Pourtant, maintenir un niveau adéquat de la matière organique des sols et le cycle biologique des nutriments est cruciale pour la réussite de la gestion des sols dans les régions tropicales humides (Tiessen *et al.* 1994; Lehmann & Rondon 2006). La matière organique est en effet primordiale pour maintenir la structure du sol et retenir les nutriments (voir ci-dessus).

Près d'un quart des terres cultivables de la planète est dégradé à force de pratiques agricoles agressives, de déforestation et d'urbanisation (Warner 1995). Les sols des zones tropicales ont subi au cours des dernières décennies un phénomène de dégradation croissante, amplifié par le changement climatique (Glaser *et al.* 2001; Lehmann 2009). De plus dans un contexte où une grande partie des agriculteurs ont peu accès aux intrants (engrais minéraux) il est important de trouver des méthodes de gestion durable et nécessitant peu d'investissements financiers. A l'inverse, une partie des sols tropicaux est cultivée suivant des méthodes modernes avec des quantités d'intrants importantes, souvent après déforestation (par exemple en Amazonie brésilienne). Ces cultures ont un rôle économique importants (produits d'exportation), mais rien ne prouve pour le moment que les pratiques actuelles (engrais minéraux, produits phytosanitaires, cultures monospécifiques) sont plus durables que les pratiques comparables utilisées dans les pays tempérés. La fragilité des sols tropicaux décrite ci-dessus suggère le contraire et il a fallu plusieurs dizaine d'années pour se rendre compte que l'agriculture moderne pratiquée dans les pays tempérés était en fait minière, c'est à dire qu'elle épuisait les ressources des sols. Dans ce contexte, il est donc très important de mettre au point de nouvelles techniques agricoles plus durables (Tilman 1998; Boody & DeVore 2006).

Ainsi, des nouvelles techniques agricoles basées sur la gestion des organismes des sols sont proposées afin d'accroître la durabilité des cultures (Lavelle & Pashanasi 1989; Beare *et al.* 1997). D'autres techniques possibles sont basées sur l'ajout de biochar au sol et la création de nouvelles "*terra preta*" (Glaser *et al.* 2002; Lehmann *et al.* 2003a; Lehmann & Rondon 2006; Glaser 2007). Ma thèse vise à participer à l'évaluation de ces deux types de technique et à tester l'utilité de les combiner: augmenter la densité de vers de terre et ajouter du biochar dans le même sol.

1.2 Effet du biochar sur la fertilité des sols et la croissance des plantes

1.2.1 Le biochar et la fertilité des sols tropicaux

Le biochar est un charbon fait à partir de biomasse végétale (néologisme anglais à partir du mot ''charcoal'' et du préfixe ''bio''). On propose maintenant l'ajout de biochar au sols tropicaux comme moyen d'augmenter le niveau de matière organique (Trujillo *et al.* 1998), d'améliorer les propriétés physiques, chimiques et biologiques du sol, et en conséquence de soutenir la fertilité des sols (Lehmann *et al.* 2003a; Steiner *et al.* 2007; Chan & Xu 2009). L'application de biochar au sol n'est pas un nouveau concept, il est devenu populaire après l'étude de sols très fertiles en Amazonie: Amazonian Dark Earths ou ''*terra preta*'', en portugais (Glaser *et al.* 2001; Lehmann & Joseph 2009b). La découverte il y a quelques années de ces terres noires très fertiles a vivement attiré l'attention et a suscité d'emblée de nombreuses études.

Ces sols apparaissent enrichis en charbon de bois fragmenté jusqu'à des profondeurs importantes (plus de 60 cm pour les sols profonds). Ces sols sont aussi enrichis en matières organiques et nutriments minéraux tels que phosphore, potassium et calcium et l'activité des microorganismes y est plus développée. Ils seraient d'origine anthropique, liés à l'activité passée de groupes humains organisés du bassin Amazonien, ayant appliqué sur de longues durées des méthodes agricoles aujourd'hui oubliées qui conféreraient aux sols une fertilité durable à l'échelle de plusieurs siècles. Ces pratiques étaient probablement basées sur une combustion incomplète et lente du bois abattu mais aussi sur le recyclage local de tous les déchets des villages. L'utilisation du biochar (''slash and char'') parait alors comme une alternative prometteuse à l'agriculture sur brûlis (''slash and burn'').

1.2.2 L'agriculture sur brûlis

Une des formes les plus commune d'agriculture en milieu tropical humide est l'agriculture itinérante sur brûlis (Glaser *et al.* 2002). Il y a environ 300 à 500 million d'agriculteurs dans les régions tropicales qui emploient cette technique d'utilisation des terres (Giardina *et al.* 2000). Brûler la biomasse aérienne relâche des nutriments minéraux qui fertilisent le sol et permettent quelques cycles de culture. Ce mode traditionnel de culture

correspond à une alternance de mise en culture de courte durée, après brulis d'une zone boisée, et d'une jachère d'une durée assez longue pour reconstituer le couvert végétal nécessaire à la restitution de la fertilité (Tiessen *et al.* 1994). Cela implique l'abandon répété de parcelles et le défrichement de nouvelles zones. De plus, ces pratiques conduisent à la destruction de forêts primaires et au dégagement de grandes quantités de gaz à effet de serre.

Le problème principal lié à la culture de l'agriculture sur brûlis est la perte rapide des éléments nutritifs du sol. Lorsqu'on brûle une forêt afin de développer l'agriculture ou l'élevage, les cultures et les herbages poussent relativement bien durant les premières années grâce aux nutriments fournis par les cendres. Mais, une fois les cendres lessivées par les pluies, la fertilité du sol est très réduite. Une fois la forêt ouverte, le sol est moins protégé par la végétation des fortes précipitations. Ces pratiques conduisent à la destruction de forêts primaires et au dégagement de grandes quantités de gaz à effet de serre. Les communautés forestières dans les régions tropicales ont pratiqué cette technique depuis des millénaires, ce qui permettrait à la forêt de se régénérer. Mais actuellement, l'augmentation de la densité des populations humaines impose une intensification de l'agriculture et rend la pratique de l'agriculture sur brûlis insoutenable (Fearnside *et al.* 2001). En effet, les périodes de jachères doivent être raccourcies et ne laissent plus au sol le temps de régénérer sa fertilité.

Cette exploitation non durable des sols contribue à fragiliser les sociétés. Dans le bassin Amazonien, indépendamment des pratiques traditionnelles, le défrichement se fait aussi dans un but spéculatif, pour développer l'élevage bovin (90% des surfaces défrichées) ou des cultures commerciales (soja). Les pâturages se dégradent généralement en quelques années imposant sans cesse la déforestation de nouvelles zones (Desjardins *et al.* 2000 ; Laurance *et al.* 2001). Pour toutes ces raisons, l'agriculture sur brûlis parait plus comme une pratique passée devant être remplacée que comme une pratique pouvant se perpétuer. La redécouverte des "*terra preta*" a remis à l'ordre du jour l'usage du biochar. En liaison avec l'agriculture sur brûlis, il s'agit de brûler la biomasse végétale disponible mais d'une manière contrôlée et lente de façon à produire du charbon de bois qui aura une durée de vie dans le sol beaucoup plus longue que les cendres.

1.2.3 La *terra preta nova*

Après les premières études sur les effets du biochar sur la fertilité des sols et sur le fonctionnement des "*terra preta*", l'idée de créer des "*terra preta*" modernes, ou "*terra*

preta nova'' est vite devenue une évidence. Il s'agirait de produire du biochar à partir de différentes sources de biomasse: copeaux de bois, bûches, bois résultant du défrichement d'une parcelle forestière, déchets végétaux, résidus de culture. La biomasse séchée est chauffée avec exclusion de l'oxygène à 400-800 °C, ce qui permet de briser les composés organiques à chaîne longue (Lehmann *et al.* 2006). Cela aboutit à une combustion partielle de la biomasse. Des études sont en cours pour optimiser la production de biochar et comprendre quelle est la meilleure façon d'appliquer le biochar (granulométrie, quantité de biochar, méthode d'application). Les premiers résultats sur l'amélioration de la fertilité avec ces techniques sont en train d'être publiés (Lehmann & Rondon 2006; Steiner *et al.* 2007; Asai *et al.* 2009; Blackwell *et al.* 2009; Chan & Xu 2009).

1.2.4 Mécanismes par lesquels le biochar affect les plantes

Le biochar augmente la capacité d'échange cationique des sols grâce à ses propriétés d'absorption (Lehmann *et al.* 2003c; Cheng *et al.* 2006; Cheng *et al.* 2008). L'addition de charbon augmente donc la capacité des sols à stocker des nutriments minéraux qui une fois adsorbés sur le charbon ont moins de chance d'être lessivés. Cela augmente finalement la disponibilité en P, Ca, Mn et Zn et N (Glaser *et al.* 2002; Lehmann *et al.* 2003a; Steiner *et al.* 2008b). Le biochar adsorbe aussi de la matière organique soluble ce qui augmente aussi la fertilité des sols.

Le biochar modifie en outre l'activité des communautés bactériennes des sols (Pietikäinen & Fritze 2000; Birk *et al.* 2008; Steiner *et al.* 2008a). Les bactéries et peut-être aussi les mycorhizes (Warnock *et al.* 2007) sont protégés des organismes consommateurs de microorganismes, comme certains protozoaires et nématodes, dans les micropores du charbon. Cela augmente alors la biomasse et l'activité de ces microorganismes qui est généralement positive pour la croissance des plantes.

Enfin, le biochar améliore la structure du sol et sa capacité de rétention d'eau (Major *et al.* 2009). Ce dernier point augmente encore la capacité du sol à retenir les nutriments puisque des ions non-adsorbés sur le biochar, en solution, ne seront pas lessivés grâce à la capacité de rétention d'eau du biochar.

Ainsi le biochar améliore toutes les caractéristiques des sols, y compris le pH des sols acides (Lehmann *et al.* 2003a; Topoliantz & Ponge 2005). Comme, de plus, la structure polycyclique aromatique du charbon de bois le rend ''récalcitrant'' aux attaques microbiennes

durant plusieurs siècles (DeLuca *et al.* 2006), l'ajout de biochar doit avoir un effet positif durable sur la fertilité des sols.

1.2.5 Avantages du biochar

L'utilisation de techniques basées sur le biochar a de nombreux avantages potentiels. Elle peut permettre de restaurer la fertilité de sols dégradés. Elle peut permettre, dans le contexte de l'agriculture sur brûlis, de maintenir la fertilité des terres défrichées pendant plus de cycles de culture et donc de freiner la déforestation. Comme le biochar augmente aussi l'efficacité de la fertilisation minérale (Steiner *et al.* 2007; Kimetu *et al.* 2008), en augmentant la rétention des nutriments, le biochar peut permettre de diminuer les quantités d'engrais appliquées. Ce qui rend alors l'agriculture plus durable puisque les engrais azotés sont produits avec des sources d'énergies non-renouvelables et que les engrais phosphorés sont extraits de gisement miniers en voie d'épuisement. On peut alors se demander si le biochar pourrait être utilisé à grande échelle dans les "cultures de rente" tropicales: canne à sucre, soja, pâturages, palmier à huile, café, cacao...etc. La durabilité de ces cultures étant souvent mise en doute, toute mesure permettant d'augmenter leur durabilité serait bienvenue.

L'autre avantage du biochar est qu'il se décompose lentement. De ce fait il améliore probablement durablement la fertilité des sols. Un sol peut alors contenir des grandes quantités de biochar et constituent alors un puits de carbone potentiellement très important quantitativement (Glaser *et al.* 2004b; Lehmann *et al.* 2006). Cela pourrait atténuer le changement climatique global en restockant le carbone atmosphérique relâché par la combustion des combustibles fossiles et les changements d'usage des sols.

1.3 Effets des vers de terre sur la fertilité des sols et la croissance des plantes

1.3.1 Les vers de terre et la fertilité des sols tropicaux

A fin de maintenir la productivité des sols dans les régions tropicales, des solutions alternatives aux techniques actuelles doivent être trouvées. Gérer la fertilité des sols en préservant la macrofaune et en particulier les vers de terre pourrait améliorer la fertilité des

sols (Lavelle *et al.* 1998). L'idée générale est que la faune du sol a un effet global positif sur la structure du sol et la dynamique de la matière organique (Lavelle *et al.* 2006) mais que les pratiques culturales ont tendances à diminuer la biomasse et la diversité de cette faune (Mäder *et al.* 2002). En milieu tropical humide, la manipulation de populations de vers de terre semble la mieux adaptée. En effet, les vers représentent généralement plus de 50% de la biomasse de la faune total dans ces milieux (Lavelle & Pashanasi 1989; Lavelle & Spain 2001) et ils exercent le plus souvent des effets positifs sur la plupart de paramètres de la fertilité des sols. De nombreux travaux mettent ainsi en évidence l'effet positif des vers sur la croissance des plantes (Brown *et al.* 1999 ; Scheu 2003; Brown *et al.* 2004a) par des mécanismes plus ou moins directes.

1.3.2 Généralités sur les vers de terre

Trois grandes catégories de vers de terre sont définies d'après des critères morphologiques, comportementaux et leur impact sur le sol (Lavelle & Spain 2001): les épigés, les anéciques et les endogés. Les endogés dominent les populations de vers dans les écosystèmes des milieux tropicaux humides (Barois 1999; Brown *et al.* 1999; Fragoso 1999). Ces vers vivent dans le sol et se nourrissent de la matière organique du sol. Pour cela, ils ingèrent de grandes quantités de sol (Lavelle *et al.* 1987) et le rejettent sous forme de turricules ce qui modifie la structure physique du sol.

1.3.3 Mécanismes par lesquels les vers de terre affectent les plantes

Les vers de terre sont connus pour leur effet positif sur la croissance des plantes (Scheu 2003; Brown *et al.* 2004a). Cinq mécanismes principaux permettent d'expliquer ces effets positifs:

(1) L'amélioration de la structure physique du sol, par le creusement de réseaux de galeries et le rejet de déjections (turricules) qui participent à la macroagrégation du sol.

Par le brassage du sol et la production de structures biogéniques (galeries et turricules), les vers modifient l'agencement des particules de sol entre elles, changeant ainsi l'agrégation, l'abondance et la distribution en classes de tailles de la porosité de ces sols. Cette modification de l'agencement des particules physiques a des conséquences sur l'équilibre entre l'eau et l'air dans les espaces poraux, ce qui conduit à une meilleure circulation

de l'air et de l'eau qui jouent un rôle important dans la croissance et la pénétration des racines (Brown *et al.* 2004a). La production des galeries par les vers accroît la macroporosité, alors que la production de turricules réduit généralement la mésoporosité au profit de la microporosité (Brown *et al.* 2004a).

(2) Les vers augmentent le recyclage des déchets organiques végétaux et de la matière organique des sols et augmentent la disponibilité des nutriments minéraux pour les plantes.

Les vers de terre peuvent accélérer directement la minéralisation de la matière organique par la consommation (vers épigés) et l'incorporation de la litière dans le sol (vers de terre anéciques) ou en ingérant directement du sol (vers de terre endogés, géophages). Les vers affectent la dynamique de la matière organique dans les sols tropicaux a différents niveaux, à l'échelle du transit intestinal ils accélèrent la minéralisation (Villenave *et al.* 1999) et favorisent la fragmentation des particules de matière organique en éléments plus fins (Lavelle *et al.* 1992). Dans les turricules frais, l'activité microbienne est stimulée (Lavelle *et al.* 1987; Barois 1999). Par la suite, la décomposition diminue de manière importante dans les turricules âgés, du fait d'une protection de la matière organique des turricules (Barois *et al.* 1987). La digestion de la matière organique a pour conséquence une libération importante de nutriments dans le sol. L'augmentation du taux de minéralisation en présence de vers de terre est particulièrement importante pour l'azote et le phosphore qui très souvent limitent la production primaire (Brown *et al.* 2004a). La minéralisation de l'azote par les vers de terre a été proposée comme principale explication de l'effet positif des vers sur les plantes.

(3) La diminution de la sensibilité des plantes à certains pathogènes/parasites comme les nématodes (Blouin *et al.* 2005)

Les vers de terre peuvent modifier l'activité des populations d'organismes nuisibles aux plantes par l'ingestion du sol, la bioturbation, la production de turricules et de galeries et la dispersion de ces organismes. Ils affectent les pathogènes de plantes en les consommant et en altérant leur distribution initiale. Cela peut conduire à une réduction des populations de ces organismes nuisibles et amoindrit la virulence de leur attaque contre les plantes.

Il a été ainsi montré que la présence des vers de terre supprime ou réduit l'incidence des maladies de plantes telles que la fonte de semis (*Pythium*) qui affecte les racines des plantules, le *Rhizoctonia* qui affecte les parties aériennes des plantes et du *Verticillium* qui provoque le flétrissement des plantes (Stephens & Davoren 1995; Chaoui *et al.* 2002). Le vermicompost peut supprimer des agents pathogènes tels que *Phytium* et *Rhizoctonia* qui affectent les parties

aériennes des plantes (Chaoui *et al.* 2002) et peut également conduire à une diminution des populations de nématodes (Arancon *et al.* 2003). Les vers de terre réduisent aussi directement, l'incidence de ravageurs tels que les nématodes en diminuant leurs populations (Senapati 1992) ou en rendant les plantes tolérantes à leur présence (Blouin *et al.* 2005) . Les dommages causés par les larves d'insectes tels que la noctuelle (*Sesamia calamistis*) foreuse du maïs sont réduits de manière importante en présence de vers de terre (Boyer 1998). De même, la présence de vers de terre (*L. terrestris*) réduit les dommages causés par la psylle (*Psylla piri*), un insecte suceur, et par la larve de la mineuse du pommier (*Phyllonorycter blacardella*) (Boyer 1998).

(4) La stimulation de bactéries (Lambrecht *et al.* 2000) productrices de phytohormones qui modifient l'allocation des ressources des plantes et augmentent leur croissance (Pasqualeto Canellas *et al.* 2002; Quaggioti *et al.* 2004).

La présence d'hormones similaires aux hormones de croissance végétale a été mise en évidence aussi bien dans les tissus de vers de terre que dans leurs turricules (Nardi *et al.* 1988). Aussi, il a été suggéré que les vers de terre participaient à la production des substances régulatrices de la croissance végétale telles que des auxines, gibbérellines ou cytokinines car ces substances ont été retrouvées en quantités significatives dans les turricules de vers de terre (Nardi *et al.* 1988). Ces substances jouent un rôle dans la multiplication et la différenciation cellulaire (Tomati *et al.* 1998; Muscolo *et al.* 1999).

Il est possible que ces substances soient produites par des microorganismes stimulés par les vers (Muscolo *et al.* 1999; Quaggioti *et al.* 2004). Il pourrait aussi s'agir d'une production directe par les vers de terre (El Harti *et al.* 2001). Que les vers soient ou non les véritables producteurs de ces substances, ils jouent un rôle majeur dans leur biosynthèse. Des extraits humiques de turricules de vers contenant des phytohormones modifient également l'activité des estérases et des peroxydases chez *Nicotiana plumbaginifolia* (Muscolo *et al.* 1993), ainsi que du glutamate déshydrogénase, la glutamine synthétase et la phosphoénolpyruvate carboxylase chez *Daucus carota* (Muscolo *et al.* 1993; Muscolo *et al.* 1996). Plus récemment, il a été montré que des substances humiques contenant de l'auxine augmentaient l'expression de gènes impliqués dans le prélèvement des nitrates chez *Zea mays*, provocant une augmentation importante de l'absorption de nitrate par la plante (Quaggioti *et al.* 2004). La production de ces hormones est à l'origine d'une augmentation de la germination des graines, de la biomasse produite et de la capacité d'absorption des nutriments par les plantes (McColl *et al.* 1982). La production de ces substances peut conduire à une modification de l'architecture racinaire des plantes. Pascualeto (2002) a en effet mis en évidence

que la présence d'acide humique (analogue à l'auxine) extraits des turricules de vers, entraîne une élongation des racines et une prolifération de racines latérales.

(5) La dispersion des organismes bénéfiques à la croissance des plantes

Les organismes tels que les mycorhizes, les protozoaires ou encore les bactéries symbiotiques favorables à la croissance des plantes ont une très faible mobilité dans le sol (Stephens *et al.* 1993). De plus, du fait des conditions environnementales du sol et du manque de ressource, une large proportion de ces organismes est inactive (en dormance) dans l'attente de meilleures conditions afin de reprendre leurs activités (Lavelle *et al.* 1997). Par leur activité de bioturbation les vers de terre mettent ces organismes au contact des ressources dont le manque limite leurs activités. Ils assurent aussi la dispersion de ces organismes aussi bien via leurs déplacements (surface du corps) que par l'ingestion/déjection du sol, ce qui permet par exemple le contact avec les racines au niveau de la rhizosphère. L'établissement des relations symbiotiques avec les plantes s'en trouve ainsi facilitée et contribue à une meilleure croissance végétale (Gange 1993; Doube *et al.* 1994).

Dans le sol, tous ces mécanismes par lesquels les vers influencent la croissance des plantes sont susceptibles d'agir simultanément. Il est difficile de déterminer l'importance relative de chacun d'eux (Brown *et al.* 2004a). De plus, en fonction de la combinaison sol-plante-ver de terre, il est difficile de prédire le sens de l'effet des vers, ou quels mécanismes sont impliqués dans des cas précis (un type de sol, une espèce de plante, une espèce de ver). Aux cinq mécanismes ci-dessus généralement avancés pour expliquer l'effet des vers, il faut rajouter deux autres mécanismes ayant des effets négatifs: la consommation des racines de plantes par les vers de terre (difficile à observer), la consommation de graines et leur enfouissement. Ce dernier mécanisme (enfouissement des graines) a une grande incidence sur la composition des communautés végétales (Grant 1983; Decaens *et al.* 2003; Milcu *et al.* 2006; Laossi 2009).

1.4 Interactions entre biochar et vers de terre

A priori, nous savons déjà que le biochar et les vers de terre sont en interaction dans le sol (Topoliantz *et al.* 2002; Topoliantz & Ponge 2003; Van Zwieten L. *et al.* 2009). Les vers ingèrent ainsi le biochar et transportent probablement les particules de biochar dans le sol. D'autre part, une partie des mécanismes par lesquels les vers et le biochar agissent sur la croissance des plantes sont de natures comparables (voir section 1.2 et 1.3) et sont donc

susceptibles d'interagir. En particulier les vers comme les terres noires augmentent la disponibilité des nutriments minéraux et augmentent la structuration du sol et sa stabilité. De plus, les vers de terre comme le biochar modifient et régulent les communautés microbiennes des sols qui jouent un rôle primordial pour leur fertilité. Enfin, certaines interactions pourraient être négatives. Par exemple, le charbon de bois pourrait adsorber une partie des phytohormones produites en présence des vers de terre, les rendant ainsi moins efficaces (Warnock *et al.* 2007). Ces possibles interactions n'ont jamais été abordées expérimentalement mais elles suggèrent que biochar et vers de terre pourraient interagir dans leur influence sur la croissance des plantes. Mon travail de thèse vise à étudier ces interactions.

Le schéma suivant (Fig. 1) décrit le cadre conceptuel de mon travail de thèse. Il détaille les différentes interactions possibles entre vers de terre, biochar et plantes. Il y a d'une part les effets directs, à court terme, des vers de terre (flèche 1) et du biochar sur la croissance des plantes (flèche 2). Il s'agit de tous les mécanismes cités ci-dessus par lesquels la croissance des plantes peut être augmentée au cours d'un cycle de vie. La flèche 5 représente les possibles interactions, à court terme, entre les mécanismes d'action des vers et du biochar: influence conjointe sur la communauté des microorganismes, influence conjointe sur la structure du sol...etc. Comme expliqué ci-dessus, les vers de terre et le biochar peuvent aussi être en interaction directe. Les vers de terre (flèche 4) peuvent ainsi ingérer le biochar, le fragmenter, l'enfouir... Ces processus pourraient participer à la maturation des *terra preta* (Ponge *et al.* 2006). On pense en effet que l'addition de biochar à la surface d'un sol n'aboutit pas instantanément à un effet positif sur la fertilité d'un sol (Steiner *et al.* 2007; DeLuca *et al.* 2009; Lehmann & Joseph 2009a). A l'inverse, le biochar pourrait avoir des effets à plus ou moins court terme sur les communautés de vers de terre (flèche 3). En changeant la capacité de rétention de l'eau et de matière organique soluble du sol le biochar pourrait par exemple changer la biomasse des vers de terre et la démographie de leur population.

En plus d'effet à court terme, l'interaction vers de terre-biochar-plantes fait aussi probablement intervenir des effets à long terme qui ne peuvent être mis en évidence au cours d'un seul cycle de végétation, par exemple dans une expérience en microcosmes. D'abord, les vers de terre (flèche 6) en jouant sur la minéralisation de la matière organique des sols et sur la rétention de nutriments minéraux peuvent avoir des effets cumulatifs dans le temps sur la disponibilité des nutriments pour les plantes (Barot *et al.* 2007b). Le biochar (flèche 7) ne joue que sur la rétention des nutriments minéraux mais cela peut aussi conduire à des effets à

long terme sur la disponibilité des nutriments en jouant sur l'efficacité du recyclage (Barot *et al.* 2007b; Boudsocq *et al.* 2009). Enfin, soit par les interactions directes entre biochar et vers (flèches 3 et 4), soit par une synergie entre biochar (diminuant les pertes de nutriments minéraux) et vers de terre (augmentant la minéralisation) aboutissant à une augmentation non-additive de la disponibilité des nutriments minéraux, (Barot *et al.* 2007b; Boudsocq *et al.* 2009) vers et biochar peuvent interagir sur le long terme dans leur influence sur la croissance des plantes (flèche 8).

Du fait des différents effets positifs des vers et du biochar sur la croissance des plantes et des interactions décrites ci-dessus, l'hypothèse générale de ma thèse est celle de l'existence d'une synergie à court et long terme entre vers et biochar aboutissant à une augmentation forte de la production de biomasse par les plantes. Une telle synergie pourrait être utilisée dans le futur par des pratiques agricoles cherchant à bénéficier à la fois des avantages des vers et du biochar.

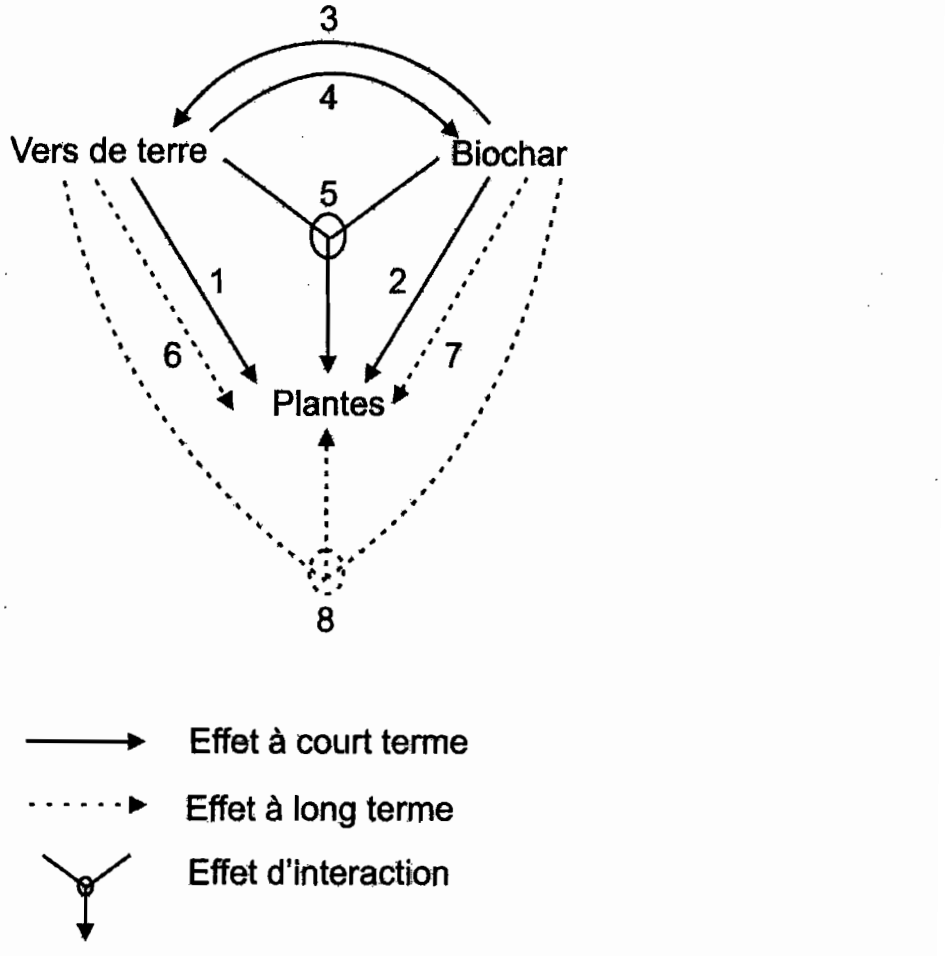

Figure 1. Schéma conceptuel des possibles interactions entre vers de terre - biochar - plante à court et a long terme

CHAPITRE 2:

OBJECTIFS ET ORGANISATION DE LA THÈSE

2 Objectifs et organisation de la thèse

2.1 Objectifs de la thèse

Ma thèse a visé à étudier les interactions de la Figure 1 au cours de diverses expériences. Bien sûr, dans la durée limitée d'une thèse, toutes ces interactions n'ont pu être étudiées aussi en détail qu'il aurait pu être souhaitable. Dans un premier temps, j'ai étudié les effets à court terme des vers, du biochar et de leur interaction (respectivement flèche 1, 2 et 5 de la Figure 1) sur la croissance du riz dans deux expériences en microcosmes. Au cours de la première expérience (Chapitre 4, première partie; section 4.1 de la thèse), j'ai comparé les effets des vers et du biochar dans deux type de sol et un troisième traitement dans lequel une fertilisation minérale a été ajoutée au sol le moins fertile. La croissance des plantes a été mesurée d'une manière précise pour décrire l'effet de différents traitements sur l'allocation des ressources des plants de riz (nombre et masses des grains, biomasse de racines, hauteur, biomasse aérienne, architecture du système racinaire, et concentration en azote des différents organes). Cette expérience visait avant tout à tester l'existence d'une synergie à court terme entre vers et biochar. La grande diversité des mesures effectuées et la comparaison entre les réponses du riz dans les trois "traitements sol" visait à mieux comprendre les mécanismes d'action des vers et du biochar. Par exemple, si l'effet des vers diminue sensiblement quand de l'engrais minéral est ajouté, cela laisse supposer que les vers n'agissent pas seulement par une augmentation de la minéralisation (Blouin *et al.* 2006).

Dans la deuxième partie du chapitre 4 (section 4.2), j'ai comparé l'effet du biochar, des vers et de la combinaison des deux facteurs sur la croissance et l'allocation des ressources de 5 génotypes de riz. L'idée de cette expérience est double. D'une part, dans une optique de développement de nouvelles pratiques agricoles basées sur l'usage du biochar et des vers de terre, il est utile de vérifier si les cultivars habituellement cultivés répondent favorablement à ces nouvelles pratiques (Zhu *et al.* 2001; McCouch 2004). Les cultivars modernes pourraient bien avoir perdu leur capacité de réponse à des acteurs naturels des sols comme les vers de terre, soit d'une manière stochastique parce que la sélection n'a jamais porté sur les traits intervenant sur cette réponse, soit parce que ces cultivars ont été sélectionnés pour pousser dans les conditions agricoles "optimales", dans des sols avec peu de matière organique, peu de vers de terre, une fertilisation minérale abondante...etc. D'autre part, dans une optique plus fondamentale, il est utile de mieux comprendre la nature de la relation plante-vers de terre. Si

on sait que la réponse de différentes espèces de plantes aux vers peut être très différente, la variabilité de cette réponse entre génotypes n'a jamais été évaluée. Cela pourrait par exemple permettre d'identifier les traits des plantes leur permettant de répondre aux vers, et en retour de mieux comprendre les mécanismes d'action des vers.

Au chapitre 5, j'aborde la réponse physiologique du riz au biochar et aux vers de terre. Il s'agit encore d'effets à court terme (flèches 1, 2 et 5) et de tester l'existence d'une interaction biochar-vers de terre. En général, les études écologiques sur les réponses des plantes à un traitement particulier du sol ont uniquement porté sur des mesures macroscopiques comme la biomasse totale et très peu d'études ont inclus des mesures physiologiques plus fines. Étudier les processus physiologiques et cellulaires induits par la présence de vers de terre, de biochar ou des deux devrait pourtant approfondir notre compréhension des mécanismes par lesquels les vers de terre et biochar influencent la croissance des plantes. Cela peut aussi être d'une manière de détecter, à un niveau plus fin, des preuves de l'interaction entre biochar et vers. L'écophysiologie moléculaire, tente d'expliquer le fonctionnement des plantes en réponse à son milieu par l'analyse des voies métaboliques et des interactions entre molécules. Les gènes (ADN) sont transcrits en ARN qui servent alors à la synthèse des protéines qui vont être les molécules opératrices dans la cellule. A chaque étape, des mécanismes de régulations interviennent et permettent à la plante de réagir aux conditions au cours du temps aux conditions environnementales dont les vers et le biochar sont un des facteurs. J'ai donc étudié l'influence du biochar et des vers de terre sur l'expression de certains gènes liés au métabolisme de l'azote et au turnover des protéines. L'idée directrice était que les vers et le biochar influencent a priori la disponibilité de l'azote minéral, et qu'ils devraient alors aussi influencer le métabolisme de l'azote au sein de la plante.

Le chapitre 6 s'intéresse à l'effet à long terme des vers de terre sur la formation de "*terre preta*" (flèches 6, 7 et 8 de la Figure 1). Ainsi, j'ai étudié l'effet du biochar et des vers de terre sur la croissance des plantes et les propriétés physico-chimique du sol dans une expérience en mésocosmes de 24 mois. Cette étude visait à estimer les conséquences à long terme des mécanismes mis en évidence dans les expériences à courte terme des chapitres 4 et 5. Pour des raisons pratiques et parce que le biochar pourrait bien être utilisé pour améliorer la durabilité des pâturages, le riz des expériences en microcosmes a été remplacé par une graminée pérenne, souvent plantée dans les pâturages sud-américains, *Brachiaria humidicola*.

Ma thèse a été complétée par un échantillonnage de terrain pour tester l'effet du biochar sur les vers de terre et le reste de la faune du sol (flèche 4 de la Figure 1). Pour cela, je me suis servi d'une expérience de terrain déjà mis en place depuis plusieurs années. Cette expérience comportait différents traitement de biochar et les traitements ''control'' correspondant. Après un échantillonnage de la macrofaune dans les différentes parcelles cela a permis de tester l'effet du biochar sur les grands groupes de la macrofaune.

Dans la dernière partie de la thèse (Chapitre 7), je discute mes résultats d'une manière globale. Le but étant de faire le bilan de mes résultats, d'interpréter conjointement les résultats des différentes expériences et de proposer des perspectives de travaux futurs. La figure 1 constitue le canevas général de ma thèse; en plus de renseigner les différentes flèches de la figure, j'ai cherché plus précisément à répondre aux questions suivantes qui sont discutées dans ce dernier chapitre: (1) Existe-t-il une synergie entre vers et biochar leur permettant d'augmenter la croissance des plantes à court terme d'une manière non-additive? (2) Les vers participent ils à la maturation de la *terra preta*, en augmentant l'effet du biochar sur le long terme? (3) La comparaison des effets des vers et du biochar sur différents génotypes et dans différents sols permet elle de préciser les mécanismes d'action respectifs des vers et du biochar sur les plantes? (4) Qu'apporte l'approche moléculaire pour répondre à cette question?

2.2 Organisation de la thèse

Dans le but de faciliter la diffusion de mes résultats dans des journaux internationaux je les présente sous forme d'articles en anglais. Ainsi, les chapitres 4, 5 et 6 sont rédigés en anglais alors que l'introduction (chapitre 1), les objectifs de la thèse (chapitre 2) une partie générale ''Matériels et méthodes'' (chapitre 3) et la discussion générale (chapitre 7) ont été rédigés en français. Dans ce ''Matériels et méthode'' général je présente d'une manière assez détaillée l'échantillonnage de terrain qui a été effectué a fin d'étudier l'effet du biochar sur la faune du sol. Cependant, je n'ai pas eu le temps d'analyser les résultats de cet échantillonnage et il n'y a donc pas de chapitre correspondant dans ma thèse.

Le chapitre 4 portant sur les effets de l'interaction entre biochar et vers de terre sur la croissance du riz. Il correspond à deux expériences en microcosmes (influence du type de sol dans le chapitre 4.1 et influence de la variété de riz dans le chapitre 4.2). Cela correspond aux articles "Contrasted effect of biochar and earthworms on rice growth and resource allocation

in different soils" qui à été soumis à Soil Biology and Biochemistry et à l'article "Contrasted responsiveness of rice cultivars to earthworms and biochar". La version présentée de ce dernier article est une première version complète mais pas encore soumise. Le chapitre 5 portant sur les effets de l'interaction entre biochar et vers de terre sur la physiologie du riz correspond à l'article: "Biochar but no earthworms enhances rice growth through increased protein turnover". Cet article est terminé et sera soumis rapidement à Journal of Experimental Botany. Finalement, le chapitre 6 portant sur les effets des vers de terre sur la formation de la terra preta correspond à l'article: "Effects of earthworm-biochar interactions on soil fertility". Cet article va encore être amélioré et sera soumis au journal Plant and Soil.

Pour les chapitres 4 à 6 la numérotation des tables et figures recommence à chaque nouvel article. La bibliographie, celle des parties en français comme celle des articles en anglais, est par contre entièrement à la fin de la thèse.

Pour finir, deux articles dont je suis coauteur sont présentés en annexe. Il s'agit de travaux de la thèse de Kam-Rigne Laossi auxquels j'ai participés :

Laossi, K.-L., D. C. Noguera, A. Bartolomé-Lasa, J. Mathieu, M. Blouin, and S. Barot. 2009. Effects of endogeic and anecic earthworms on the competition between four annual plants and their relative reproduction potential. Soil Biol. Biochem **41**:1668-1773.

Laossi, K.-L., A. Ginot, D. C. Noguera, M. Blouin, and S. Barot. 2009. Earthworm effects on plant growth do not necessarily decrease with soil fertility. Plant Soil in press.

Il s'agit encore de l'effet des vers de terre sur les plantes, mais abordant cette fois l'influence des vers sur la démographie des plantes et la compétition entre espèces de plantes. Par contre, l'influence du biochar n'y est pas du tout abordée.

CHAPITRE 3:

MATÉRIEL ET MÉTHODE

3 Matériel et méthode

A fin de mieux comprendre les effets de vers de terre et du biochar sur le sol et sur la croissance des plantes, nous avons développé une approche expérimentale en serres en microcosmes et mésocosmes. Un échantillonnage de terrain de la macrofaune a aussi été conduit. Ce chapitre réunit des informations générales sur la méthode utilisée pour ces différents travaux. Un numéro a été attribué à chaque expérience:

Expérience I en microcosmes:

a) "Effets de l'interaction entre biochar et vers de terre sur la croissance du riz". (Influence du type de sol dans le chapitre 4.1).

b) " Effets de l'interaction entre biochar et vers de terre sur la physiologie du riz" dans le chapitre 5.

Expérience II en microcosmes:

" Effets de l'interaction entre biochar et vers de terre sur la croissance du riz" (Influence de la variété de riz dans le chapitre 4.2).

Expérience III en mésocosmes:

" Effets des vers de terre sur la formation de la terra preta" dans le chapitre 5.

Expérience IV sur le terrain:

"Effet de la ''*Terra preta*'' sur la faune du sol "

3.1 Généralités

3.1.1 Présentation générale des sites

Les expériences I a, I b, II et III ont été conduites dans les serres à la station de recherche du Ciat (Centro Internacional de Agricultura Tropical) située en Cali (Colombie) (N 3° 31' 76'', W 76° 19'). La température moyenne est de 25°C et l'humidité relative se maintient autour de 60-70 % tout au long de l'année.

L'expérience IV de terrain a été conduite dans la ferme à Matazul situé dans la région de savane des Llanos Orientales en Colombie (N 04° 10' 15.2", W 36° 07' 12,9"). La température moyenne y est de 27°C. La pluviométrie moyenne annuelle est de 2200 mm et 95% des précipitations se situe entre avril et décembre. Une saison sèche marquée se produit donc entre janvier et mars.

3.1.2 Les sols

Les essais expérimentaux pour les microcosmes et les mésocosmes ont été réalisés sur sols provenant de Matazul (Llanos Orientales) et Pescador (Cauca) en Colombie.

Le sol utilisé pour les expériences Ia, Ib, II et III provient de la localité de Pescador (N 2° 8', W 76° 33'). Le sol de Pescador est volcanique. Il est classé comme inceptisol. Ce sol est moyennement acide (pH (H_2O) = 5.1), il est relativement riche en matière organique (MO 11,5 %) et en azote minéral (12,9 mg NH_4^+ N kg^{-1}, 27 mg NO_3^- kg-N^{-1}). La CEC est relativement élevée (6.0 cmol kg^{-1}) et la texture est dominé par l'argile (sable 24.06 %, lime 27.56 % et argile 48.38 %). La densité du sol est 0,8 g cm^{-3}.

Nous avons aussi utilisé le sol provenant de Matazul pour l'expérience Ia. C'est un oxisol (Rippstein *et al.* 2001). Ce sol est légèrement plus acide que celui de Pescador (pH (H_2O) = 4.3). Le teneur en matière organique et en azote minérale est beaucoup plus faible que pour le sol de Pescador (MO 5.22 % ; azote minéral 4,32 mg $NH4^+$ N kg^{-1}, 7.39 mg, $NO3^-$-N kg^{-1}). La CEC est relativement faible (3.0 cmol kg^{-1}). Le sol a une texture equilibrée à dominante légerment sableuse (sable 41.23 %, argile 34.39% and lime 23.78%) (Gijsman *et al.* 1997). La densité du sol est de 1.30 g cm^{-3}

Le sol pour les différentes expériences a été prélevé jusqu'à 10 cm de profondeur. Avant utilisation et la mise en microcosmes et mésocosmes, le sol a été séché à l'air et tamisé à 2 mm.

3.1.3 Le biochar

Le biochar utilisée pour l'expérience IV a été préparé au CIAT selon le protocole décrit par Rondon (2007). Le biochar a été produit à partir de troncs de "Matarraton" (*Gliricida sepium*) utilisant un grand four à température contrôlée. La température a été

maintenue à 350 °C, et l'oxygène a été maintenu à 15%. Le biochar a été broyé pour passer au travers d'un tamis de 0,9 mm.

3.1.4 Les vers de terre

Les vers de terre, de l'espèce *Pontoscolex corethrurus* (Muller 1857) utilisés dans toutes les expériences ont été collectés à Pescador (Cauca, Colombie). *P. corethrurus* est une espèce endogée à distribution tropical de la famille Glossoscolecidae originaire du plateau Guyanais (Righi 1984). Cette espèce est généralement présente dans les zones perturbées par l'activité humaine. L'activité des adultes est généralement concentrée dans les 10 premiers cm du sol, riches en matière organique mais peu atteindre des zones plus profondes en période sèche (Fragoso *et al.* 1997). Comme toute les espèces endogées, elle ingère du sol et arrive à en assimiler une partie de la matière organique non-figurée (Barois *et al.* 1993; Lavelle 1999).

P. corethrurus vivre dans conditions édaphiques d'acidité élevée, cette espèce a une grande tolérance aux caractéristiques physico-chimiques des sols, aux conditions de température et de humidité, une assimilation efficace de matière organique de faible qualité et une aptitude à la colonisation facilitée pour une démographie très active (Lavelle *et al.* 1987). Ce vers affecte les propriétés physiques du sol par la construction de macropores et de turricules qui constituent généralement des macroagrégats stables. Ces constructions ont un effet significatif sur la macro-porosite du sol, sur l'infiltration de l'eau et sur l'agrégation (Lavelle *et al.* 1987; Lavelle *et al.* 1992).

3.1.5 Le riz

Les différentes variétés de riz utilises dans notre étude ont été fournies par CIAT (Colombie). Le riz *Oryza sativa* est une plante de la famille des Poacées (graminées). Le riz est le première ressource alimentaire de la humanité et le riz a été choisi car est une espèce annuelle qui peut réalise la totalité de son cycle en 3 à 4 mois, ce qui convient dans les expériences en microcosmes qui sont généralement de courte durée. Le riz est une espèce idéal pour étudier l'impact des vers car c'est un des plants annuelles qui répond le mieux a la présence de vers de terre (Brown *et al.* 1999), le riz a l'avantage d'avoir le génome entièrement séquencé, de ce fait les études moléculaires et cellulaires sont facilités.

Finalement, le riz est une des cultures locales les plus développées et les plus étudiées par l'Unité des Ressources Génétiques du CIAT à Cali.

Dans les différentes expériences on a utilisé 5 cultivars de riz: un cultivar de riz africain (*Oryza glaberrima*, IRGC 103544) et quatre cultivars de riz asiatique (*Oryza sativa*): *Linea 30* (CIRAD 409), *Azucena* (IR64), *Nipponbare* (IRGC 12731) et "*Donde lo tiren*".

O. Glaberrima a été sélectionné et cultivé dans les régions d'Afrique occidentale depuis plus de 3500 ans. Il a développé des mécanismes adaptatifs pour supporter les stress biotiques et abiotiques.

Linea 30 a été conventionnellement développé par hybridation. Cette variété est bien adaptée aux savanes de la Colombie et *Linea 30* a été longuement utilisée depuis les années 90.

Azucena est un type de demi-nain dérivé du croisement intensif de lignes améliorées, *Azucena* a un bon rendement potentiel et elle est résistante aux nombreux stress biotiques.

Nipponbare est un cultivar japonais qui a été utilisé comme modèle génotypes de riz (il a été séquencé). C'est le résultat de sélection successif sur les variétés du Japon et se caractérise par une bonne croissance.

Donde lo tiren est une variété rustique de la Colombie.

3.1.6 Le pâturage

Le pâturage *Brachiaria humidicola* (Rendle) utilisée dans l'expérience 3 des mésocosmes est une graminée fourragère rampante dépassant rarement 50 cm de haut. Cette graminée pérenne est souvent plantée dans les pâturages colombiens et elle est origine africaine.

3.2 *Protocoles spécifiques et plans d'expérience*

3.2.1 Expérience I a en microcosmes: "Effets de l'interaction entre biochar et vers de terre sur la croissance du riz" (Influence du type de sol dans le chapitre 4.1).

La première expérience en microcosmes a été réalisée durant l'été 2006 au CIAT de Cali. Les microcosmes sont des pots de PVC (10 cm de diamètre 10 et 15 cm de hauteur). Ils ont été remplis avec 900 kg de sol. Le sol a été maintenu à 80 % du capacité de champ.

Les sols utilisés ont été récolté en juillet 2006 durant la saison des pluies, à partir de deux experiences à long terme. La premier expérience a débuté en 2004 dans une plantation de café à Pescador (Colombie). La deuxième expérience a débuté en 2002 dans un pâturage de Matazul (Colombie). Les deux expériences visaient à évaluer l'effet du biochar au champ et comportaient donc un traitemen "biochar" et un traitement "control". Les sols ont été récoltés dans ces deux traitements pour les traitements correspondant de mon expérience en microcosmes. Pour chaque microcosme, dans les traitements "vers", cinq adultes de *Pontoscolex corethrurus* ont été introduite. Deux jours après l'introduction des vers de terre, 5 grains de riz ont été semés (Oryza sativa cv. Linea 30, Chatel *et al.* 2003). Une semaine plus tard, une seule plante a été conservée dans chaque microcosme.

Les plants de riz ont été soumis aux quatre combinaisons possibles des facteurs "biochar" et "vers". Toutes les combinaisons de traitements ont été implémentées dans trois traitements sol (voir ci-dessous). Cinq répétitions ont été mises en place pour chaque combinaison de traitement. 60 microcosmes ont donc été mis en place. Le riz a été cultivé en serres pendant trois mois.

Le sol de Pescador est nommé (R) en raison de sa plus grande qualité agronomique et le sol Matazul est nommé (P) pour sa plus grande pauvreté en nutriment et matière organique. Un dernier traitement sol a été implémenté dans notre expérience. Il s'agit du sol "riche" de Pescador auquel de l'engrais a été rajouté (P+F) à trois reprises au cours de l'expérience (NPK 20, 20 et 40 kg ha^{-1}). N a été fourni sous forme d'urée.

À la fin du cycle de l'expérience les plantes ont été récoltées. Nous avons récolté la biomasse aérienne totale, la biomasse racinaire et la biomasse des grains. Les différentes biomasses ont été séchées dans un four électrique à 60° C pendant 2 jours. Le contenu de ces

biomasses en C et N a été mesuré avec un CHN. En outre, au moment de la récolte, un échantillon de sol a été prélevé dans chaque microcosme pour effectuer des analyses chimiques: nitrate et ammonium, C et N total. Le système racinaire a été analysé en utilisant un système d'analyse d'image numérique (WinRHIZO, version 2003b, Instrument Regent, Québec, Canada). Enfin, toujours en fin d'expérience, les vers de terre ont été récupérés, comptés et pesés.

Les données ont été traitées par des méthodes univariées classiques (ANOVA testant l'effet des vers de terre, du biochar et du sol ainsi que toutes les interactions entre ces facteurs).

3.2.2 Expérience I b en microcosmes: "Effets de l'interaction entre biochar et vers de terre sur la physiologie du riz" dans le chapitre 5

Cette expérience en microcosmes a été réalisée à partir des feuilles de riz de l'expérience I a. Pour cette étude on a seulement utilisé le sol de Pescador (R).

Nous avons déterminé l'effet des vers de terre, du biochar et de leur interaction sur l'expression d'un groupe de gènes liés à l'homéostasie cellulaire. Ce sont des gènes codant pour les quatre classes d'endoprotéases (protéase à cystéine, protéase à sérine, protéase à acide aspartique et métalloprotéases) ainsi que leurs inhibiteurs naturels. En effet, l'équilibre entre l'expression des gènes codant pour les endoprotéases et ceux codant pour leurs inhibiteurs est déterminant pour l'équilibre homéostasie cellulaire. Il a été démontré que certaines contraintes environnementales font basculer cet équilibre vers un catabolisme plus important (El Maarouf *et al.* 1999; Cruz de Carvalho *et al.* 2001). Ce catabolisme, plus ou moins important selon les variétés de plantes et les conditions environnementales, serait lié, au recyclage de l'azote et permettrait de remobiliser de l'azote pour synthétiser de nouvelles protéines. Cela a été testé en étudiant l'expression de gènes primordiaux de l'anabolisme de l'azote: ceux codant pour la Rubisco. D'autre part, l'augmentation de l'expression de gènes d'endoprotéases peut être considérée comme un marqueur de stress. En effet, si les traitements "*terra preta*" et vers de terre influencent significativement la disponibilité en azote ceci pourrait conduire à une diminution du niveau de stress et devrait se traduire par des modifications de l'expression des gènes cités. Cette approche est entièrement novatrice. Elle vise à faire le lien entre l'échelle écologique et l'échelle moléculaire, ce qui est très rarement réalisé.

Prélèvement du matériel végétal

Pour notre étude, nous avons prélevées des feuilles de riz avant la floraison. Trois feuilles sont prélevées et immédiatement congelées dans l'azote liquide. Les feuilles de riz ont été finement broyées dans de l'azote liquide à l'aide d'un mortier et la poudre obtenue a été conservée à - 80°C.

Extraction et dosage des ARN totaux

L'extraction des ARN totaux foliaires a été faite au moyen du Rneasy Midi kit de Qiagen selon les instructions du fabricant. Les ARN totaux ont été élus dans l'eau DEPC (1 g/L) qui provoque l'inhibition des ARNases. Les échantillons d'ARN sont dosés par spectrophotométrie à une longueur d'onde de 260 nm avec Nanodrop ND-1000 spectrophotometer.

Le design des amorces

Plusieurs amorces, ont été produites directement à partir de séquences d'endoprotéases connues du Riz, disponibles dans la base de données du web (GenBank): protéases à acide aspartique (*Oryzasin*, D32144 et *OsAP1*, D12777); protéases à cystéine (*CystP1*, X80876 et *OsCatB*, AY916493); protéases à sérine (*OsSP1*, AB037371 et *OsSP2*, AY683198) et deux gènes codant pour les sous-unités de rubisco (*rbcS*, D00643 et *rbcL*, L24073).

Réaction de PCR

Les ARN totaux du Riz ont été extraits et puis ont été convertis en ADNc par une réaction de retro-transcription. Les ADNc ont ensuite été amplifiés par PCR en utilisant le couple d'amorces choisit. La réaction de PCR utilise comme matrice les ADNc correspondants aux transcrits (ARNm) présents dans chaque traitement différent (avec ou sans vers de terre). Elle permet de détecter des différences d'expression des gènes étudiés entre chaque traitement (vers / sans vers, charbon / sans charbon). Trois répétitions ont été effectuées pour chaque traitement pour tester statistiquement la significativité des résultats.

L'activité protéolytique

L'étude de l'activité protéolytique correspondant à chaque classe d'endoprotéase a été effectuée à partir d'extraits protéiques provenant d'échantillons de riz soumis à différents traitements. Les extraits protéiques ont été extraits par broyage des feuilles conservées à - 80°C avec un tampon Tris-HCl (0,5M, pH 6,8) suivit d'une centrifugation 1 minute à 14500 t/min, transfert du surnageant dans un tube eppendorf (1.5 ml) et nouvelle centrifugation à

14000 t/min pendant 20 minutes à 4°C. Les protéines solubles présentes dans le surnageant ont été dosées selon la méthode de Bradford (1976). La mesure de l'activité protéolytique a été effectuée en utilisant le sérum albumine bovine (SAB) comme substrat sous différents pH (50 µg d'extrait protéique avec 10 µl de SAB 10% dans un volume final de 100 µl, 16 h à 37°C). La réaction a été arrêtée avec 100 µl de TCA 10% froid et après une centrifugation (15 minutes à 14000 rpm) l'absorbance (correspondant à l'hydrolyse de la SAB) a été mesurée à λ=280 nm à l'aide du Nanodrop.

Mesures complémentaires

Des mesures complémentaires comme la mesure des teneurs en azote, de la biomasse des racines et des feuilles, de la teneur en chlorophylle, de la surface foliaire ont été effectuées comme indicateurs complémentaires de l'état physiologique du riz. Le protocole de récolte d'échantillons pour ces mesures complementaires est le même que pour l'expérience I a. Les analyses de nitrate, ammonium et de C/N sur des échantillons sol et matériel végétal ont été effectuées la même manière que dans l'expérience I a.

3.2.3 Expérience II en microcosmes: "Effets de l'interaction entre biochar et vers de terre sur la croissance du riz" (Influence de la variété de riz dans le chapitre 4.2)

Pendant l'été 2007 la seconde expérience en microcosmes a été réalisée au CIAT, en Colombie. Les microcosmes sont de pots de PVC. Ils ont été remplis de 1500 g de sol sec provenant de Pescador. Le sol Pescador de cette expérience correspond au sol qui a été utilisé pour l'expérience I a en microcosmes. Cette fois-ci, 5 varietes de riz ont été utilisées: *Oryza glaberrima*, IRGC 103544, *Linea 30* (CIRAD 409), *Azucena* (IR64), *Nipponbare* (IRGC 12731) et "*Donde lo tiren*" (cultivar local colombien).

Pour chaque microcosme, cinq adultes de *Pontoscolex corethrurus* ont été introduits. Deux jours après l'introduction des vers de terre, 5 grains de riz ont été plantés Après 1 semaines, une seule plante a été conservés dans chaque microcosme. Les plantes ont été soumis aux quatre combinaisons possibles de deux facteurs: avec et sans vers et avec et sans biochar. Pour chaque combinaison de traitements (E x B x riz cultivar), deux traitements de

fertilisation ont été mis en œuvre: sans ou avec fertilisation minérale . Cinq repetitions ont été mis en œuvre pour chaque traitement, résultant en 200 microcosmes.

Après seize semaines, les plantes ont été récoltées et séparés en grains, feuilles et tiges. Le protocole de récolte d'échantillons (sol, matériel végétal et vers de terre) et d'analyse est le même que pour l'expérience I a.

3.2.4 Expérience III en mésocosmes: "Effets des vers de terre sur la formation de la *terra preta*"

L'expérience a été effectuée dans des mésocosmes durant l'été 2006. Il s'agit de bacs de 40 kg de sol sur 50 cm de profondeur. Les mésocosmes ont été installés dans une serre ouverte sur les côtés mais protégeant de la pluie au Ciat en Colombie. L'expérience a été maintenue pendant 2 ans.

Le sol a été introduit dans les bacs en suivant le protocole mis au point par Marco Rondon pour créer des terres noires (Rondon *et al.* 2007) . Les bacs ont été remplis par 40 kg de sol provenant de Pescador. Le sol a été tamisé à 2 mm et séché à l'air avant d'être utilisé pour les mésocosmes. Ensuite, le biochar a été ajouté au sol dans la moitié des mésocosmes. Le biochar, tamisé à 2 mm, a été mélangé aux 5 premiers centimètres de sol dans les traitements biochar. La même espèce de vers que pour les expériences en microcosmes a été utilisée (*Pontoscolex corethrurus*) pour les traitements vers. Les vers ont été initialement introduits dans chaque mésocosme des traitements "vers". Une touffe de *Brachiaria humidicola* a été plantée dans chaque mésocosme.

Quatre combinaisons de traitements de deux facteurs ont été mis en place: avec vers / sans vers et avec ajout de biochar/sans ajout. Pour chaque combinaison de traitements deux traitements de fertilisation ont été mis en œuvre: sans ou avec fertilisation minérale. En faisant 5 répétitions par combinaison de traitements j'ai donc installé 40 mésocosmes.

Tout au long de l'expérience, nous avons vérifié que les vers de terre se sont maintenus dans les traitements "avec vers" (présence de turricules à la surface du sol). Tous les 3 mois, la production de biomasse aérienne a été récoltée, séchée, pesée, et replacée au deux tiers dans les mésocosmes pour pouvoir évaluer l'effet des traitements sur la production végétale.

L'expérience s'est terminée durant l'été 2008. Nous avons récolté la biomasse aérienne totale et la biomasse racinaire. J'ai aussi prélevé des échantillons de sol à plusieurs profondeurs (0-5, 5-10, 10-20 et 20-30 cm). Les échantillons de sol prélevés ont été séchés à l'air avant de faire les analyses de teneur en nitrate, ammonium, carbone et azote total à fin de tester l'effet des vers de terre sur les principales variables jouant un rôle dans la fertilité des sols. Enfin, les vers de terre ont été récupérés, comptés, pesés et identifiés.

Une expérience de ''drainage'' a aussi été réalisée: une même quantité d'eau a été versée dans chaque mésocosme. La quantité d'eau traversant le sol et ressortant par les trous de drainage des mésocosmes a été mesurée pour évaluer les effets des traitements sur la capacité de rétention en eau du sol. La teneur en nitrate et ammonium de cette eau a été mesurée.

Les données ont été traitées par des méthodes univariées classiques (ANOVA testant l'effet des différents facteurs et leurs interactions).

3.2.5 Expérience IV sur le terrain:" Effet de la "*Terra preta*" sur la faune du sol"

Parcelles expérimentales

Le dispositif expérimental qui a servi à réaliser cette étude à été mis en place par le CIAT et CORPOICA à Matazul dans la région de savane des Llanos Orientales (Colombie). (N 04°10'15.2", W 07 °36'12.9"). Ce dispositif expérimental a eu lieu sur des oxisols.

Les parcelles ont été établies sur le même type de sol (oxisol Rippstein *et al.* 2001) mais avec des différents types d'usage des sols:

- Parcelles cultivées sous un système de culture conventionnelle sous rotation des cultures de mais (*Zea mays).* - soja (*Glycine max*), avec un labour classique.
- Parcelles de savane naturelle (pâturages natifs) ou aucun labour n'est réalisé.
- Parcelles semées de pâturage.

Dans les différentes parcelles mentionnées ci-dessus, deux types de traitement ont été établis: l'ajout ou non de biochar au sol. Le traitement biochar a été mis en place en décembre 2002. L'application de biochar a été faite une seule fois dans les différentes parcelles (parcelles agronomiques) à un taux d'application de 8 et 20 t ha $^{-1}$. Des parcelles témoin non

enrichies en charbon ont aussi été établies dans les trois mêmes types d'usage des sols. Cette expérience avait été mise en place par M. Rondon en 2002 (Tableau 1). 3 parcelles (répétitions) pour chaque combinaison de traitement ont été installées.

Culture	Forte application de biochar
	Faible application de biochar
	Control
Savane native	Forte application de biochar
	Faible application de biochar
	Control
Savanes avec pâturage	Forte application de biochar
	Faible application de biochar
	Control

Tableau 1: Combinaison des différents facteurs appliqués dans l'expérience.

Echantillonnage de la macrofaune

L'échantillonnage pour évaluer l'effet de la terra preta sur la macrofaune a été réalisé durant l'été 2006. Chaque parcelle du dispositif expérimental a été échantillonnée.

L'extraction de la faune du sol a été réalisée en utilisant la méthode TSBF (Tropical soil Biology and fertility) (Lavelle & Pashanasi 1989; Anderson & Ingram 1993). Pour chaque parcelle, un monolithe de sol de 25 cm de côté et 30 cm de profondeur a été prélevé au centre de la parcelle. Un cadre de 25 cm de côte est utilisé pour marquer l'emplacement du monolithe, qui est isolé en creusant une tranche de 20 cm de large tout autour. Après avoir récupéré la litière, on découpe le monolithe en 3 couches successives de 10 cm d'épaisseur. La faune est triée et séparée manuellement sur le terrain.

La faune a été classée en grandes groupes (vers de terre et autres) au laboratoire sur du matériel fixé; conservation dans de l'alcool à 75% pour l'autre faune et fixation du formol à 4 % pour les vers de terre.

La macrofaune

Les macro-invertébrés trouvés dans les 27 parcelles ont été d'abord classés en grands groupes: les vers de terre, les fourmis, les termites et les coléoptères. La plupart de la faune du sol a été classée et identifiée au niveau de famille. Les principales familles rencontrées sont: *termitidae, formicidae, coccidae, tenebrionide, carabidae, sthaphylinidae* et *blatellida.*

Pour les vers de terre, la détermination a été effectuée au niveau de famille. Les vers de terre étaient principalement localisés dans l'horizon 0-20 cm avec une préférence pour l'horizon 0-10 cm. Aucun vers a été trouve dans la litière sol. Les termites et les fourmis sont retrouvés dans tous les horizons des sols. Elles sont bien représentées dans tous les types d'usage des sols.

Les données de cet échantillonnage n'ont pas été encore analysées. Les données seront traitées par des méthodes multivariées (description globale de la communauté dans les différentes cultures et les deux types de sol) et des méthodes univariées classiques (ANOVA à deux facteurs et une interaction, testant l'effet du type de sol, de la culture et de l'interaction sur la densité des différents groupes ou espèces).

CHAPITRE 4.1:

EFFETS DE L'INTERACTION ENTRE BIOCHAR ET VERS DE TERRE SUR LA CROISSANCE DU RIZ

INFLUENCE DU TYPE DE SOL: "CONTRASTED EFFECT OF BIOCHAR AND EARTHWORMS ON RICE GROWTH AND RESOURCE ALLOCATION IN DIFFERENT SOILS"

4 Effets de l'interaction entre biochar et vers de terre sur la croissance du riz

4.1 *Influence du type de sol: "Contrasted effect of biochar and earthworms on rice growth and resource allocation in different soils"*

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4.1.1 Abstract

Addition of biochar to soils and increase in earthworm biomass are potential ways to increase the fertility of tropical soils and the sustainability of crop production in the spirit of agroecology and ecological engineering. However, a thorough functional assessment of biochar effect on plant growth and resource allocations is so far missing. Moreover, earthworms and biochar increase mineral nutrient availability through an increase in mineralization and nutrient retention respectively and are likely to interact through various other mechanisms. They could thus increase plant growth synergistically.

This hypothesis was tested for rice in a greenhouse experiment. Besides, the relative effects of biochar and earthworms were compared in three different soil treatments (a nutrient rich soil, a nutrient poor soil, a nutrient poor soil supplemented with fertilization).

Biochar and earthworm effects on rice growth and resource allocation highly depend on soil type and are generally additive (no synergy). In the rich soil, there are both clear positive biochar and earthworm effects, while there are generally only positive earthworm effects in the poor soil, and neither earthworm nor biochar effect in the poor soil with fertilization.

The analysis of earthworm and biochar effects on different plant traits, confirms that they act through an increase in nutrient availability. However it also suggests that another

mechanism, such as the release in the soil of molecules recognized as phytohormones by plants, is also involved in earthworm action. This mechanism could for example help explaining how earthworms increase rice resource allocation to roots and influence the allocation to grains.

Key-words: agroecology, ecological engineering, *Oryza sativa*, plant growth, *Pontoscolex corethrurus*, resource allocation, shoot/root ratio, soil type.

4.1.2 Introduction

Managing soil fauna (especially earthworms Lavelle *et al.* 2001) and biochar applications (Lehmann *et al.* 2003c; Glaser 2007) are often proposed as appealing ways to increase the fertility of tropical soils in a sustainable way. Indeed, tropical soils are often poor in organic matter (Tiessen *et al.* 1994) and tend to have low cation exchange capacitites (Glaser 2007) and both earthworms and biochar influence soil organic matter dynamics, the release of mineral nutriments and their retention. While studying the effect of biochar on plant growth is a fairly new field of (Lehmann & Rondon 2006; Steiner *et al.* 2008b; Blackwell *et al.* 2009), effects of earthworms on plant growth is an old field. Nevertheless, this issue has mostly been addressed in terms of biomass accumulation and more seldomly in term of resource allocation (Scheu 2003; Laossi 2009). Our study aims at meeting this need and particularly at determining the effect of biochar and earthworms on plant resource allocation and at infering the underlying mechanisms. Moreover, comparing biochar and earthworm effects, which influence soil properties and plant growth partially (and only partially) through the same mechanisms, should throw new lights on this broad suject.

The application of biochar, i.e. incompletely combusted organic matter, (Glaser *et al.* 2002; Lehmann *et al.* 2003c) is historically not a new practice. It has re-emerged after the study of the *Terra Preta do Indio,* which are highly fertile soils (Lehmann *et al.* 2003c). The soils were created by Amerindian populations in pre-Columbian times (Glaser *et al.* 2002). Apart from high SOM contents, the most striking feature of *Terra Preta*; is their high nutrient content (Glaser 2007). This suggests that creating modern *Terra Preta* could be a way to increase tropical soil fertility and to maintain higher soil carbon stocks, thus mitigating the current rise in atmospheric CO_2. (Marris 2006). Biochar can enhances long-term soil fertility through several mechanisms. The polycyclic aromatic structure of biochar makes it chemically and biologically stable, allowing it to persist in the environment for centuries

(DeLuca *et al.* 2006). Besides this remarkable chemical structure, biochar has a porous physical structure which leads to very large surface area (Lehmann & Rondon 2006). This increases the soil cation exchange capacity as well as its capacity to retain dissolved organic matter (Lehmann & Rondon 2006). Moreover, biochar modifies the community of soil microorganisms as well as the activity of these microorganisms, probably because it provides a suitable habitat for them (Pietikäinen & Fritze 2000). This is likely to improve directly and indirectly plant growth (Reynolds *et al.* 2003; Marris 2006).

Maintaining high biomasses of earthworms would be another sustainable way to increase tropical soil fertility (Lavelle *et al.* 2001). Two reviews about the effect of earthworms on plant growth (Brown *et al.* 1999; Scheu 2003) showed that plant shoot biomass is higher in the presence of earthworms (70–80% of the reviewed experiments). Five mechanisms have been shown to be involved in these positive effects (Brown *et al.* 2004a): (1) increased mineralization of soil organic matter therefore increasing nutrient availability; (2) production of plant growth substances via the stimulation of microbial activity; (3) biocontrol of pests and parasites; (4) stimulation of symbionts and (5) modification of soil porosity and aggregation which induces changes in water and oxygen availability to plants.

Manipulating earthworms and soil content in biochar are two ways to manipulate soil fertility in the spirit of agroecology and ecological engineering. Indeed, in the two cases, soil physicochemical and biotic characteristics are modified interactively through ecological processes and a parsimonious use of external imputs. Biochar and earthworms influence plant growth through mechanisms that are partially the same: they both change soil structure and soil microbial community (Pietikäinen & Fritze 2000; Brown *et al.* 2004a) and influence nutrient cycling. While, earthworms increase organic matter mineralization on the short term (Scheu 2003; Brown *et al.* 2004a), biochar increases the retention of mineral nutrients (Lehmann & Rondon 2006) which decreases lixiviation and is likely to increase nutrient availability on the long term (Lehmann *et al.* 2006; Lehmann & Rondon 2006). Finally, biochar and earthworms have been shown to directly interact: earthworms ingest biochar particles and reject them in their casts, which is likely to influence biochar distribution in the soil profile (Topoliantz & Ponge 2003; Topoliantz *et al.* 2005). Therefore, we hypothesize that earthworms and biochar interact in the ways they influence plant growth. To test this hypothesis and to compare the respective effect of earthworms and biochar we investigated, in a greenhouse microcosm experiment, the effects of earthworms (*Pontoscolex corethrurus)* and biochar on rice growth (*Oryza sativa*).

It has already been shown that earthworm effects on plant growth change with soil type (Doube *et al.* 1997; Wurst & Jones 2003; Brown *et al.* 2004a; Laossi *et al.* 2009a; Laossi 2009) but the effect of biochar on plant growth across different soil types has never been directly studied. Therefore, in the present work, each treatment (eathworm and biochar) was implemented in three different soil treatments: two unfertilized soils of contrasted fertility, and the lower-fertility soil supplementted with mineral fertilizer. Assessing the responsiveness of crops to biochar and earthworms in different soils and according to agricultural practices is indeed required to determine where and when using biochar and earthworms improves crop sustainability. This should also help infering the underlying mechanisms (Blouin *et al.* 2006; Laossi *et al.* 2009a).

Finally, the effect of earthworms and biochar on total plant biomass production has been studied much more than their effects on plant resource allocation. We thus also analyzed the way earthworms and biochar influence the allocation to seeds, roots and shoots, root system architecture and allocation of nitrogen. This is for example usefull to determine whether earthworms and biochar increase crop yield (here, the total grain biomass) or only increase the accumulation of vegetative biomasses. This should alos give insights on the mechanisms through which earthworms and biochar influence plants. Altogether, the following questions were specifically addressed: (1) What is the relative magnitude of biochar and earthworms on rice growth? (2) Does the rice responsiveness to earthworms and biochar change with soil treatments? (3) Do earthworms and biochar interact in the way they influence rice growth? (4) How do earthworms and biochar modify rice resource allocation?

4.1.3 Materials and methods

Experimental design

The experiment was conducted at CIAT (Centro Internacional de Agricultura Tropical) greenhouses in Cali, Colombia. Plants were submitted to the four possible combinations of two factors: with and without earthworm (respectively noted E, NE) and with and without biochar (respectively B, NB). All treatments combinations were implemented in three soil treatments (see below). Five replicates were implemented for each treatment, resulting in 60 microcosms. Rice was grown in greenhouses for three months under controlled conditions: relative humidity= 65-95%, temperature= 27-29°C, light intensity = 600 umol.m^{-2} s^{-1} and a 12 h photoperiod.

Microcosms

Experiment containers (microcosms) consisted of PVC pots (diameter 10 cm and 15 cm height). They were filled with 900 g) of sieved (2 mm) dry soil. Drains at the bottom of pots were covered with 1 mm plastic mesh to prevent earthworms from escaping. Soil was maintained at 80% soil field capacity (checked through regular weighing of the pots).

Microcosms were arranged in a completely randomized design.The soil was collected in july 2006, during the rainy season, from two long term field experiments that aimed at comparing plant production in plots with and without the addition of biochar: an experiment on coffee which was established in 2004, in the Andean hillsides of the Cauca Department, south-western Colombia (Pescador, 2° 48'N 76° 33' W), second, an experiment on grass production which was established in 2002 (Matazul, 4°19'N, 72°39'W in the Colombian Eastern Planes, Llanos). Soil was collected in the control treatments of these experiments for our microcosm treatments without biochar, and from their biochar treatments for our microcosm treatments with biochar. In this case, the soil contained respectively 25.5 and 45.5 g of biochar per dry kg of soil for "Pescador" and "Matazul". The soil was collected in the 0-10 cm layer.

The Pescador soil is a volcanic-ash soil, an inceptisol, in the USDA classification system (USDA, 1998). This soil is called hereafter the rich soil (R) because of its higher agronomic quality. The soil is moderately acid (pH (H_2O) = 5.1). It is relatively rich in organic matter MO (11.5 %), and mineral nitrogen (12.9 mg NH_4^+-N kg^{-1}, 27 mg NO_3^- -N kg^{-1}). The CEC is relatively high (6.0 cmol kg^{-1}). Texture is dominated by clay (24.06% sand, 27.56% silt, and 48.38% clay). The soil bulk density is 0.8 g cm^{-3}.

The Matazul soil is a clay–loam oxisol, therefore a poor soil (P) which has developed from alluvial sediments (Rippstein *et al.* 2001) . It is hereafter refered to this soil as the poor soil (P) because it is less fertile that the inceptisol. This soil is slightly more acid (pH (H2O) = 4.3) than the R soil. The contents in organic matter (5.22 %) and mineral nitrogen (4.32 mg NH4 +-N kg-1, 7.39 mg, NO3– -N kg-1) are much lower than in the R soil. The soil has a low capacity to retain cations (CEC 3.0 cmol kg-1). The soil has a rather equilibrated texture (41.23% sand, 23.78% silt and 34.39% clay). (Gijsman *et al.* 1997). The bulk density in the native savanna is 1.30 g cm–3 (Trujillo *et al.* 1998) . A last soil treatment (P+F) was implemented in our experiment. It consists in the soil of the grassland oxisol to which fertilizer was added in our microcosms. The P+F treatment received three times a NPK treatment (20, 20 and 40 kg ha-1, respectively for P, K and N). N was provided as urea. We

use the terms "poor soil" and "rich soil" to facilitate the comprehension of our article. However, the two soils are very different and differ by many other caracteristics than their content in organic matter and mineral nutrients. These caracteristics are also likely to influence the effects of biochar and earthworms on plant growth (see the Discussion).

For each microcosm, five adults (initial total fresh weight 4.0 +/0.5 g) of *Pontoscolex corethrurus* (Annelida: Oligocheata: Glossoscolecidae), were introduced. Earthworms were collected at the Pescador site (Cauca, Colombia).. Two days after introducing earthworms, 5 rice seeds (Oryza sativa cv. Linea 30, Chatel *et al.* 2003) were sown. After 2 weeks, only one seedling was kept in each microcosm and these were regularly weeded during the experiment (other seedlings were removed).

Measurements

After ten weeks, plants were harvested and separated into grains, leaves, and stems. Roots were collected by wet sieving. Fresh root systems were scanned and analysed using a digital image analysing system (WinRHIZO, version 2003b, Regent Instrument, Quebec, Canada). Each plant part was dried in an electric oven at 60°C for 2 days. Subsamples of each plant material were analyzed for total carbon, total nitrogen and C/N. The parameters considered were: total biomass, shoot biomass, root biomass, total grain biomass, total root length, mean root diameter, shoot root ratio, root ramification, mean grain weight, grain number, grain C/N, root C/N, leaf C/N and total N content in rice. At harvest time, a final soil sample was collected for chemical analyses. Nitrate and ammonium were quantified colorimetrically using a segmented flow analyzer (Skalar Autoanalyzer, Skalar, The Netherlands).

Statistical analysis

ANOVAs were implemented using the SAS GLM procedure (sum of squares type III, SS3) (SAS 1990). A full model was first used to test all possible factors: Biochar (B), earthworms (E) and soil (S) ("B", "E", and "S") and all, two-fold and three-fold, interactions between these factors (see Appendix 1). Since significant interactions between biochar and soil and earthworms and soil were detected, data were reanalysed separately for each soil (Table 1, 2, 3 and 4). To determine the direction of significant effects, we used multiple comparison tests based of least square means (hereafter LSmeans, LSmeans SAS statement), taking into account the Bonferroni correction. Presenting separate models for each soil treatment allows displaying the results in a more pedagogic way.

4.1.4 Results

For all ANOVAs the R^2 values are very high, i.e. above 0.70 in most cases, and above 0.90 in many cases (see Table 1 to 4). This shows that a high percentage of the variable variability can be explained by the treatments. The percentages of variation given below are relative to the control tretment and correspond all to significant LSmeans comparisons.

Soil content in nitrate and ammonium

Biochar and earthworms tend to increase both the nitrate and the ammonium content of the soil in the three soil treatments (Table 1, Fig. 1). However, in the R soil, earthworms increase soil mineral nitrogen content (+92 % for nitrate and +80 % for ammonium) more than biochar (+44% for nitrate and no significant effect on ammonium). Conversely, biochar does not affect soil nitrate content in the P+F soil treatment. Biochar and earthworm effects are most of the time additive, but there are also some exceptions. For example, in the P soil, both biochar and earthworms increase soil content in nitrate and ammonium, but the combination of the two does not increase further this content. Conversely, in the fertilization treatment, soil ammonium content decreases with earthworms (-7%), increases with biochar (+14%), and increases more in the presence of both biochar and earthworms (+40%).

Table 1. ANOVA table of F values for the effects of biochar (B) and earthworms (E) and their interaction on nitrate and ammonium soil concentrations. The last line gives the significant LS mean differences between treatment combinations. One model has been analysed for each soil: R, rich soil; P, poor soil; P+F, poor soil + fertilizer. Total df =15. *, p<0.05; **, p<0.01; ***, p<0.001; NS not significant.

		R		P		P+F	
	df	Nitrate	Ammonium	Nitrate	Ammonium	Nitrate	Ammonium
B	3	**19.20***	**10.88***	**25.75****	**414.18*****	2.27 NS	**60.71*****
E	3	**75.46*****	**45.62*****	**22.10****	**261.13*****	**21.24***	**5.91***
E*B	3	0.18 NS	**4.61*****	**12.53****	**499.24*****	1.63 NS	**17.32***
R^2		0.922	0.884	0.883	0.993	0.758	0.912
LS means		**EB>E>B>C**	**EB>E>B>C**	**EB,B,E>C**	**EB,B,E>C**	**EB,E>B, C**	**EB>B,E,C**

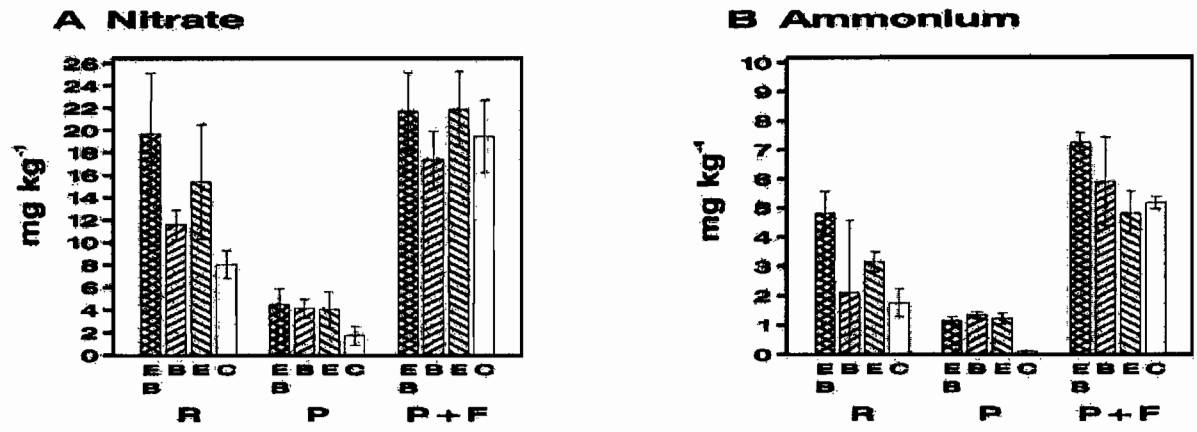

Fig.1. Effects of biochar and earthworms on: (A) nitrate and (B) ammonium soil concentrations. Mean values are displayed together with standard deviations. R, rich soil; P, poor soil; P+F, poor soil + fertilizer. EB, earthworm and biochar; E, earthworm; B, Biochar; C, control. Significant differences between means are marked by different letters (least square means).

Total biomasses and allocation to reproduction and roots

The shoot and total biomasses are higher in the R and P+F soils than in the P soil (Fig. 2). Root biomass and total grain biomass are comparable in the two P soil treatments and higher in the R soil. There is a significant effect of E and B in most cases. The interaction between E and B is only significant for the total biomass, root biomass and total grain biomass in the P soil and for the root biomass in the P+F soil. There is no significant effect on the shoot/root ratio in the P soil and on the total biomass and the shoot biomass in the P+F soil (Table 2).

The total and shoot biomasses follow the same pattern. In the R soil, the effect of E and B are both significant but the effect of B (+147 and +166%) is stronger than the effect of E (+82 and +98% respectively). The two effects are additive (no interaction). In the P+F soil, there is no significant effect on the total and shoot biomasses. In the P soil, the clearer effect is a positive E effect (+167 and +200% respectively). It must be highlighted that the combination of E and B allows reaching the same total biomasses as the ones obtained in the P+F soil.

Root biomass follows a different pattern than the shoot and total biomasses. In the three soils, the stronger effect is a positive E effect (Fig. 2). In the P+F soil the pattern is more complicated due to a significant interaction between B and E.

The total grain biomass follows another original pattern. In the R soil there are positive E (+92%) and B (+294%) effects. In the P soil there is an interaction between E and B leading to a +800% increase in total grain biomass in the E and B treatment. In the P+F soil there is only a negative E effect on the total grain biomass, (-21%).

Table 2. ANOVA table of F value for the effects of biochar (B) and earthworms (E) and their interaction on Total biomass, Shoot biomass, Root biomass and Total grain biomass. One model has been analysed for each soil: -R, riche soil; -P, poor soil; P+F, poor soil + fertilizer. Total df = 19. *; **; *** $p<0.05$, $p<0.01$, $p<0.001$, NS not significant.

		R			
	df	**Total biomass**	**Shoot Biomass**	**Root biomass**	**Total grain Biomass**
B	1	**213.04*** **	**191.44 *** **	0.19 N.S.	**174.37*** **
E	1	**75.28*** **	**78.31 *** **	**13.75*** **	**25.96*** **
E*B	1	1.69 N.S.	1.82 N.S.	0.29 N.S.	2.00 N.S.
R^2		0.95	0.94	0.470	0.92
LS means		**EB>B>E>C**	**EB>B>E>C**	**E>B,C**	**EB>B>E>C**

		P			
	df	**Total biomass**	**Shoot biomass**	**Root biomass**	**Total grain biomass**
B	1	**20.74*** **	4.42 N.S.	**10.79 *** **	**38.06*** **
E	1	**138.32*** **	**96.11*** **	**87.20*** **	**12.82** **
E*B	1	**6.23 ***	0.61 N.S.	**5.37 ***	**13.93** **
R^2		0.91	0.86	0.86	0.80
LS means		**EB,E>B,C**	**EB,E>B,C**	**EB, E>B, C**	**EB>B,E,C**

		P+F			
	df	**Total biomass**	**Shoot biomass**	**Root biomass**	**Total grain biomass**
B	1	0 NS	1.71 N.S.	**36.98*** **	0.14 N.S.
E	1	2.31 N.S.	0.42 N.S.	**96.10 *** **	**11.92 ***
E*B	1	1.49 NS	0 N.S.	**15.23 ** **	1.28 N.S.
R^2		0.19	0.12	0.90	0.45
LS means		**NS**	**NS**	**E>EB>B E>C**	**B>EB**

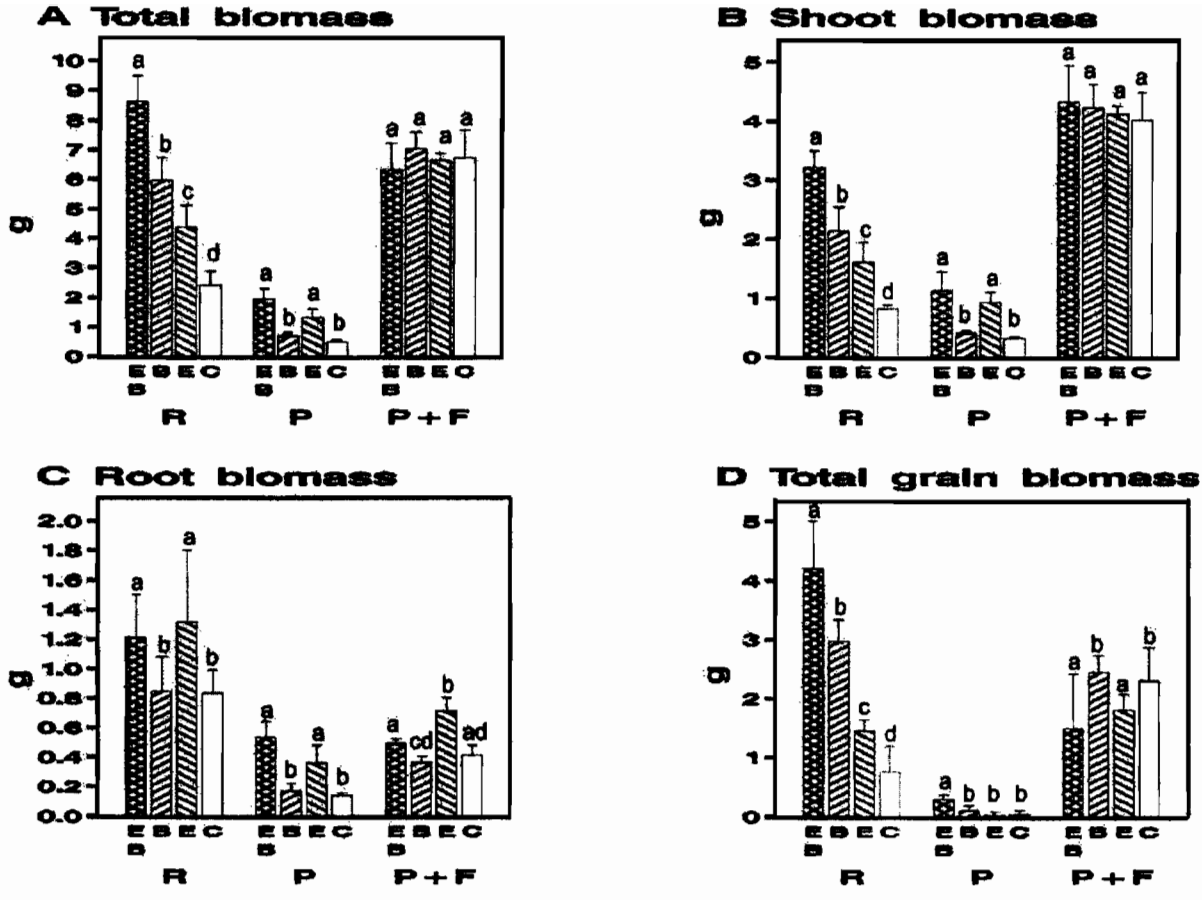

Fig.2. Effects of biochar and earthworms on: (A) Total biomass, (B) Shoot biomass, (C) Root biomass, and (D) Total grain biomass. Mean values are displayed together with standard deviations. R, rich soil; P, poor soil; P+F, poor soil + fertilizer. EB, earthworm and biochar; E, earthworm; B, Biochar; C, control. Significant differences between means are marked by different letters (least square means).

Allocation of resources within the aerial and root systems

All variables but root ramification are influenced by the soil treatment (Fig. 3). In general, variables take higher values in the P+F soil. Differences between the R and P soils tend to be less clear. However, the mean grain weight and the grain number are higher in the R than in the P soil. Besides, the mean grain weight is comparable in the P and P+F soils but higher in the R soil. There is a significant interaction between E and B in the R, P and P+F soils. Overall, the stronger effect is the effect of earthworms which is positive in the R and P soils and negative in the P+F soil (Table 3 and Fig. 3).

The total root length and the mean root diameter follow roughly the same pattern (Fig. 3 and Table 3, LSmeans comparisons). In all soil treatments, the E and B treatments lead to root systems with higher total root length and root diameter. Then, the effect of B determines the ranking between the four treatment combinations. B effect tends to be positive in R and P soils, and is not significant in the P+F soil. In the R and P soils, B effect increases the total root length (+1.6% and + 19% respectively) and the mean root diameter (+56% and +4% respectively). The number of ramifications by unity of root length (root ramification) presents a higher variability within treatments than other root related variables (Fig. 3). However, for the R and P+F soils, E has a positive effect (+22% and 32% respectively) on root ramification, while B has a negative effect (-24% and -13% respectively, Fig. 3). In the P soil there is only a significant positive E effect on root ramification (+34%).

The shoot/root ratio follows a different pattern from the root biomass. In particular the E and B effects are different in the three soils. In the R soil there is only a positive effect of B (+220%) on the shoot/root ratio, i.e. decrease in the proportion of resources allocated to roots. In the P soil there is no significant effect of the treatments. In the P+F soil there is a positive B effect (+20%) and a negative E effect (-45%) on the shoot/root ratio (which leads to a complex ranking of treatments as described by LSmeans, Table 3).

Grain production and grain filling were very limited in the P soil. This leads to small and very variable values for the mean grain weight and the grain number (Fig. 3). There is no significant effect on the mean grain weight in the R soil (Table 3). However, in this soil, there is a significant strong positive E (+55 %) and B (+232%) effect on the grain number. In the P soil, there is no significant effect on the mean grain biomass, but positive E (+150%) and B effects (+250%) on the grain number. Finally, in the P+F soil there is a negative E effect on the mean grain weight and no significant effect on the grain number.

Table 3. ANOVA table of F value for the effects of biochar (B) and earthworms (E) and their interaction on Total root length, Mean root diameter, Shoot root radio, Root ramification, Mean grain weight and Grain number. One model has been analysed for each soil: -R, riche soil; -P, poor soil; P+F, poor soil + fertilizer. Total df = 19. *; **; *** $p<0.05$, $p<0.01$, $p<0.001$, NS not significant

		R					
	df	**Total Root length**	**Mean root diameter**	**Shoot-root Radio**	**Root ramification**	**Mean grain weight**	**Grain Number**
B	1	**13.47****	**10.06****	**122.58 *****	**8.42 ****	1.29 N.S.	**178.34*****
E	1	**33.91*****	**6.97 ***	0.69 N.S.	**15.23 ****	2.76 N.S.	**17.71*****
E*B	1	**11.81****	0.41 N.S.	0.25 N.S.	0.51 N.S.	0.63 N.S.	1.75 N.S.
R^2		0.78	0.52	0.88	0.60	0.22	0.92
LS means		**EB,>B,E,C**	**EB>C**	**EB,B>E,C**	**EB,E >B**	**NS**	**EB>B>E>C**

		P					
	df	**Total Root length**	**Mean root diameter**	**Shoot-root Radio**	**Root ramification**	**Mean grain weight**	**Grain number**
B	1	**29.89*****	**10.17****	0.66 N.S.	1.32 N.S.	2.97 N.S.	**18.56 *****
E	1	**54.71*****	**35.10 *****	0.10 N.S.	**12.32 ****	1.09 N.S.	**12.89 ****
E*B	1	**18.22*****	**8.15***	0.99 N.S.	0.19 N.S.	0.76 N.S.	6.31 N.S.
R^2		0.86	0.76	0.41	0.46	0.22	0.70
LS means		**EB>B,E,C**	**EB>B,E,C**	**NS**	**EB, E>B,C**	**NS**	**EB>B,E,C**

		P+F					
	df	**Total Root length**	**Mean root diameter**	**Shoot-root Radio**	**Root ramification**	**Mean grain weight**	**Grain number**
B	1	2.08 N.S.	1.78 N.S.	**10.99 ****	0.91 N.S.	1.44 N.S.	0.29 N.S.
E	1	**11.3****	2.33 N.S.	**50.96 *****	**36.82 *****	**13.61 ****	0.79 N.S.
E*B	1	0.98 N.S.	0.78 N.S.	0.4 N.S.	1.25 N.S.	3.73 N.S.	0.15 N.S.
R^2		0.47	0.23	0.79	0.70	0.53	0.07
LS means		**EB, E>B, C**	**NS**	**B>EB, E C>E**	**EB, E>B, C**	**B, C>EB, E**	**NS**

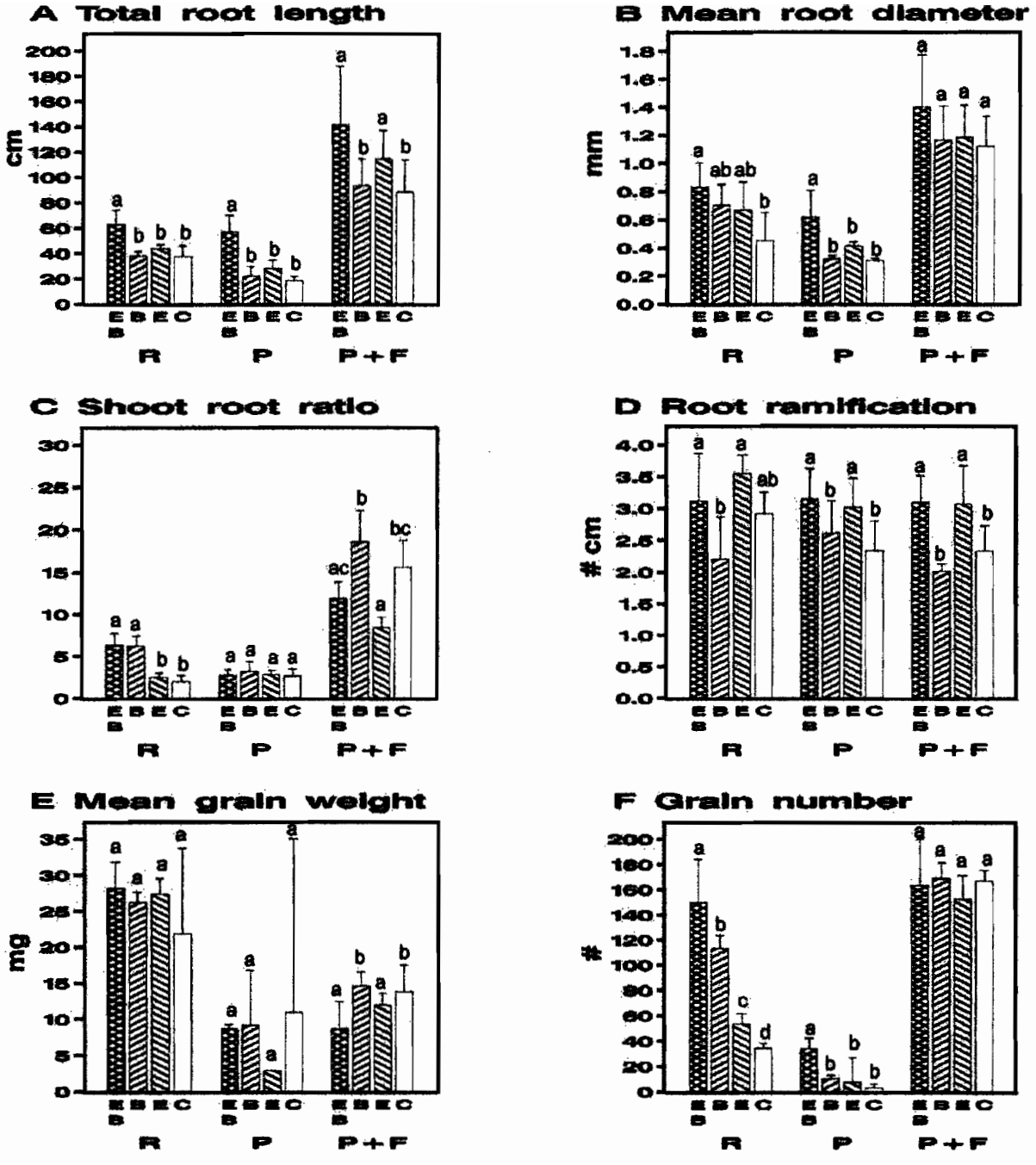

Fig.3. Effects of biochar and earthworms on: (A) Total root length, (B) Mean root diameter, (C) Shoot root ratio, (D) Root ramification, (E) Mean grain weight and (F) Grain number. Mean values are displayed together with standard deviations. R, rich soil; P, poor soil; P+F, poor soil + fertilizer. EB, earthworm and biochar; E, earthworm; B, Biochar; C, control. Significant differences between means are marked by different letters (least square means).

C/N ratios and total nitrogen content

C/N does not markedly differ between soil types. However, the total nitrogen content was higher in R and P+F than in the P soil (Fig. 4). Earthworms tend to have a negative effect on the C/N ratio of grain, root and leaf C/N in the three soils (Fig. 4). This effect was significant for the root (-9%) and leaf C/N (-40%) in the R soil. For the leaf C/N, there is a negative E effect (-32%) in the P soil. In the P+F soil there is a negative E effect on the grain, root and leaf C/N (-18%, -15% and -47 % respectively Table 4). Additionally, in the R soil there is a positive B effect on the grain, root and leaf C/N, (+22%, +109% and +45% respectively). The only significant interaction between earthworm and biochar was for the grain C/N in the P+F soil (-27 % in the B and E treatment).

Treatment effects on the total nitrogen content highly depend on the soil treatment (Table 4). In the R soil, biochar significantly increases the total nitrogen content (it is multiplied by a factor 3). Earthworms increase the total nitrogen content in the three soil treatments. There is no significant interaction between earthworm and biochar for the total nitrogen content.

Table 4. ANOVA table of F value for the effects of biochar (B) and earthworms (E) and their interaction on Grain C/N, Root C/N, Leaf C/N and Total N content. One model has been analysed for each soil: -R, riche soil; -P, poor soil; P+F, poor soil + fertilizer. Total df =15 for Grain C/N, Leaf C/ N, Root C/N and Total N content in R soil. Total df =3 for Grain C/N, and Total N content in P soil. Total df =15 for Root C/N, and Leaf C/ N in P soil. Total df =15 for Grain C/N, Leaf C/ N, Root C/N and Total N content in P+F soil. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NS not significant.

		R			
	df	Grain C/N	Root C/N	Leaf C/N	Total N content
B	1	**7.52 ***	**155.53*** **	**10.61 ** **	**34.08*** **
E	1	20.13 NS	**13.22** **	**19.28 *** **	**83.70.*** **
E *B	1	1.53 N.S.	5.34 N.S.	0.61 N.S.	2.93 N.S.
R^2		0.70	0.93	0.72	0.90
LS means		**EB,B>E,C**	**B>EB>E,C**	**B>EB,E**	**EB>E>B>C**

		P			
	df	Grain C/N	Root C/N	Leaf C/N	Total N content
B	1	-	2.27 N.S.	3.04 N.S.	0.36 NS.
E	1	-	1.19 N.S.	**10.92 ** **	10.60 NS
E *B	1	-	3.80 N.S.	0.39 N.S.	0.23 NS
R^2		-	0.37	0.54	0.56.
LS means		-	**NS**	**B, C>EB, E**	**NS**

		P+F			
	df	Grain C/N	Root C/N	Leaf C/N	Total N content
B	1	0.10 N.S.	0.01 N.S.	0.18 N.S.	0.31 N.S.
E	1	**46.42*** **	**7.94***	**34.34 *** **	**60.61 ** **
E *B	1	**4.53 ***	0.05 N.S.	0.06 N.S.	1.33 N.S.
R^2		0.80	0.39	0.74	0.83
LS means		**B,C>EB,E**	**NS**	**B,C>EB,E**	**B, C>EB, E**

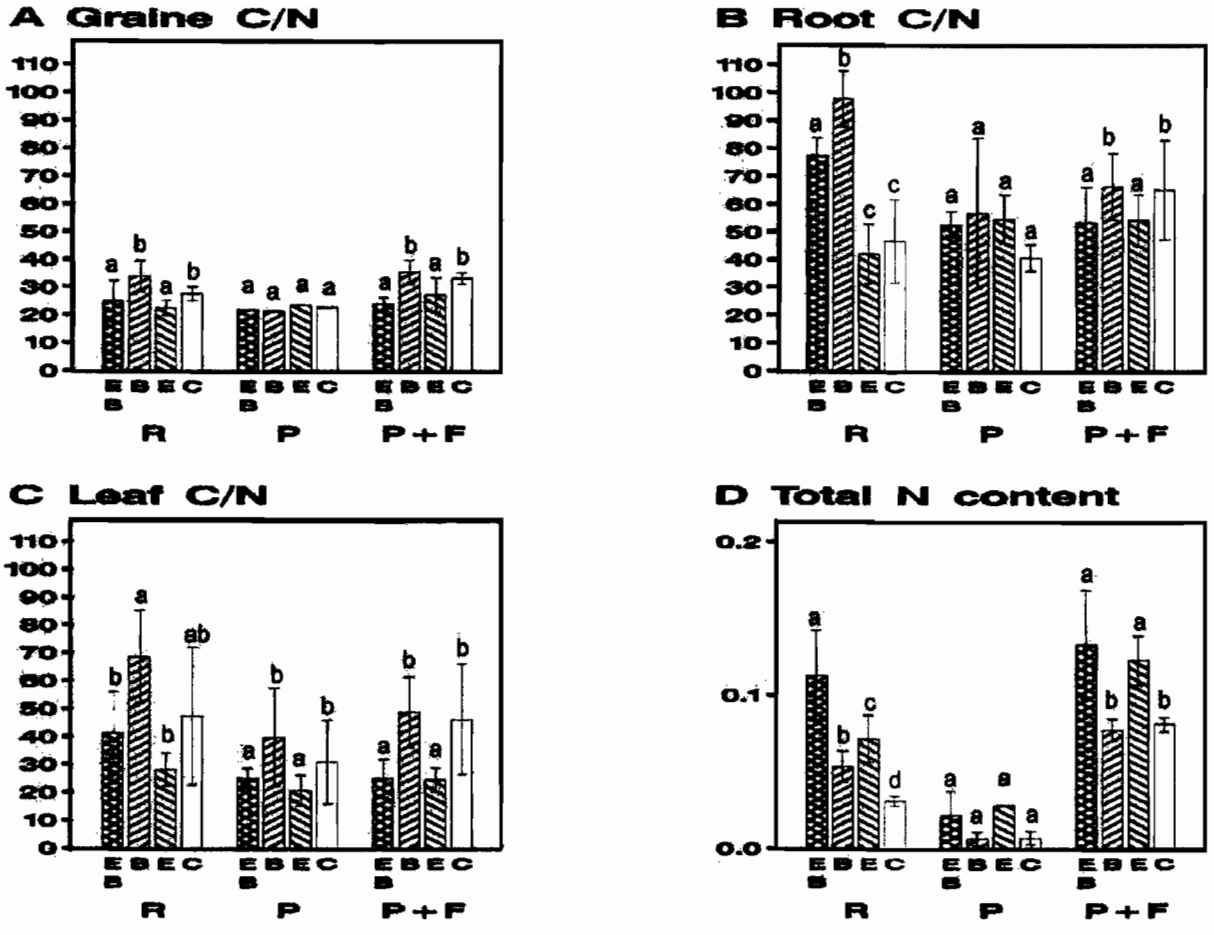

Fig.4. Effects of biochar and earthworms on: (A) Grain C/N, (B) Root C/N, (C) Leaf C/N and (D) Total N content. Mean values are displayed together with standard deviations. R, rich soil; P, poor soil; P+F, poor soil + fertilizer. EB, earthworm and biochar; E, earthworm; B, Biochar; C, control. Significant differences between means are marked by different letters (least square means).

4.1.5 Discussion

In our study, there are more significant effects of earthworms than significant effects of biochar on rice growth and related traits (first question in the introduction). However this is due to the fact that earthworms and biochar effects depend on the soil type (second question). While there are many significant effects of earthworms and biochar in the R soil, there are mostly significant earthworms effects in the P soil. In the same vein, the fertilization of the P soil tends to decrease the amplitude of earthworms and biochar effects. However, in this

fertilization treatment, there are significant earthworm effects on some plant traits and significant biochar effects for other traits. Contrary to our expectation few significant effects of the interactions between earthworms and biochar on plant traits were found (third question). The different plant traits present different patterns of responsiveness to earthworms and biochar. For example; traits describing the root system, the C/N of the different plant parts and the total biomasses responded differently to earthworms and biochar. Moreover, we have shown that the shoot/root ratio was directly impacted by earthworms and biochar. These results show that earthworms and biochar affect the resource allocation of rice plants (forth question). Linking results on different plant traits, we interpret below our detailed results and infer from them informations on the underlying mechanisms.

Effects on soil mineral nitrogen

The fertilization treatment involved the addition of urea. This led to a clear increase (about five fold) in both nitrate and ammoium soil concentration. This shows that urea quickly mineralized. Earthworms increased the availability of nitrate and ammonium in the three soil treatments as usually found in earthworm experiments (Lavelle & Spain 2001; Bhattacharya & Chattopadhyay 2004; Amador & Görres 2005) This should be due to several mechanisms: microbial activity is stimulated in earthworm young casts (Lavelle 1999; Brown *et al.* 2000; Chaoui *et al.* 2003) and earthworms mix and framgment the organic matter they ingest without assimilitaing it (Lavelle 1999). The fact that the absolute increase in mineral concentration in the presence of earthworms was the same in the poor soil and the poor soil with fertilization suggests that earhtworm effect in the fertilization treatment was only due to stimulation of the mineralization of the same amount of organic matter and not to an interaction with the addition of urea. Biochar globally increased mineral nitrogen concentration in our experiment. This should be due to the fact that biochar increases the CEC and thus increases the retention of cations such as ammonium (Lehmann *et al.* 2003a; Lehmann *et al.* 2003b). The positive effect of biochar on nitrate concentration could be achieved through a retention of water by biochar particles (Glaser *et al.* 2002). However, biochar did not increase the concentration of nirate in the fertilization treatment. This could be due to a saturation of the soil by the nitrate coming from the quick mineralization of urea and the subsequent nitrification. Finally, earthworms and biochar alone increased mineral nitrogen concentration in the poor soil but the combination of the two did not increase futher this concentration contrary to what was observed in the rich soil. It is not possible to conclude on

the underlying mechanism but interactions between earthworms and biochar are possible, for example due to their respective effects on microbial biomass and the consecutive nutrient immobilization by microorganisms.

Contrasted effects of biochar and earthworms in the three soil treaments

Brown (2004a) has remarked that the response of plants to earthworms should depend on the type of soil and it is generally assumed that positive effects of earthworms on plant growth are more likely in poor soils than in rich soils (Doube *et al.* 1997; Brown *et al.* 2004b). The rationale behind this hypothesis is that earthworms mostly affect plants because they increase the mineralisation of soil organic matter. Hence, if a soil is naturally rich in mineral nutrients or if it is fertilized artificially, earthworm effect should be diluted and could disappear (Blouin *et al.* 2006). In our case, earthworms increased the total biomass in the poor and rich soils but did not have this effect when the poor soil was fertilized. This effect of fertilization supports the above mentioned hypothesis and suggests that *P. corethrurus* first increased rice total biomass through an increased mineralization. Although earthworms also increased the soil content in ammonium and nitrate in the fertilization treatment, the impact of this increase was somehow diluted by fertilization.

Rice response to biochar depended on soil type. The positive effect of biochar on the total biomass was not observed in the poorer soil. Moreover this positive effect was no longer observed in the poor soil when it was fertilized. Using the same rationale than for earthworms (see above), this suggests that biochar mostly affected rice growth because of its positive effect on nutrient retention and availability. That biochar clearly increased the availability of mineral nitrogen in the two soils but not in the fertilization treatment supports further this rational. The absence of biochar effect in the P soil is more difficult to explain. Indeed, field experiments showed that the same biochar treatment in the same soil has a positive effect on maize biomass (+140 % , Major, unpublished results of the field experiment from which our soil, with or without carbon, has been extracted) and the concentration of biochar was higher in the P than in the R soil. Moreover, biochar increased soil content in nitrate and ammonium in the P soil (see above). A possible explanation would be that P soil being intrinsically poorer in mineral nutrients than the R soil, biochar effects is lower in the P soils because there are less nutrient to immobilize. More generally, other mechanisms through which biochar influences soil properties and plant growth are likely to interact with soil properties. This is

the case for biochar effect on soil structure and soil capacity to retain water. This is also the case for the modifications of soil microbial communities by biochar.

Lack of interaction between biochar and earthworms

There are were very few significant interacting effects between biochar and earthworms on plant traits. This suggests that the mechanisms through which earthworms and biochar increased rice growth did not interact. This is especially meaningful, in the R soil where there was a positive biochar and earthworm effect for most plant traits measured. In this soil, earthworm effect is supposed to be due to an increase in mineralization and biochar effect is supposed to be due to a decrease in leaching (see above). These two mechanisms could act in synergy, the first providing more mineral nutrient to the soil, the second helping to maintain them in the soil. That such a synergy was not observed is our experiment could be explained by the short duration of the experiment. Indeed, in the field, earthworms could build up the stock of nutrients immobilized in biochar year after year, while in our experiment biochar probably increased the initial stock of mineral nutrients and earthworms just triggered a new flux of mineral nutrient that was immediately absorbed by rice roots. Biochar effect on nutrient retention could indeed be more influential over whole vegetation cycles along which plants and their roots are likely to be inactive during a part of the time, thus allowing for more nutrient leaching. Moreover, on the long term, earthworms could also influence nutrient retention which could interact further with biochar, either positively or negatively (Subler *et al.* 1997; Domínguez *et al.* 2004; Sheehan *et al.* 2006; Barot *et al.* 2007b).

Effects on roots

Earthworms increased root biomass in the poor soil, as already reported in other earthworm experiment implemented in nutrient poor soils (Welke & Parkinson 2003; Wurst *et al.* 2003). In such cases, it is usually assumed that earthworms foster mineralization and thus the availability of mineral nutrient, as was found in our experiment. Plant would then increase their root biomass to take advantage of this increased resource availability (Wurst *et al.* 2004). It is more surprising that *P. corethrurus* also increased root biomass in the rich soil and in the fertilized poor soil. In the rich soil, earthworms increased the total biomass and the availability of mineral nitrogen, which suggests (as explained above) that their effect on mineralization was strong enough to increase nutrient availability and to impact plant growth

despite the originally high availability of mineral nutrients in this soil. The addition of fertilizer to the poor soil suppressed the effect of earthworms on the total biomass, but did not suppress their effect on root biomass. This could first be due to the fact that earthworms increased the availability of mineral nitrogen in the fertilization treatment, and that the subsequent nutrient availability might not have reached the threshold above which root biomass no longer respond to nutrient availability. Alternatively this could be due to the fact that plant investment to their root system depends both on the global availability of mineral nutrients and on their local distribution in the soil (Ingestad & Agren 1991; Bell & Sultan 1999; Farrar *et al.* 2003). Here, fertilization globally increased nutrient availability but earthworm activities are likely to increase locally the availability of mineral nutrients, in their casts (Lavelle & Martin 1992; Lavelle 1999). This could in turn stimulate locally the root production (Fitter 1976; Wurst *et al.* 2003) which could increase the total root biomass in spite of the globally high nutrient availability. Finally, earthworms are known to trigger the release of molecules recognized as phytohormones by plants (Muscolo *et al.* 1999). In particular, they have been shown to trigger the release of auxin-like molecules that increase root biomass (Pasqualeto Canellas *et al.* 2002). This could explain the maintenance of an increase in root biomass in the rich soil and with fertilization. Besides, it cannot be excluded that these two mechanisms are not also involved in the observed increase in root biomass in the presence of earthworms in the two unfertilized soil treatments.

Biochar is known to modify soil physicochemical parameters (Glaser *et al.* 2002; Lehmann *et al.* 2003a; Lehmann *et al.* 2003b), which is likely to affect root biomass and architecture but has so far never been described. In all our soil treatments, and contrary to earthworms, biochar did not lead to an increase in root biomass. This could be due to the fact that biochar effect on the availability of mineral nitrogen is less clear than earthworm effect (no effect on ammonium in the rich soil, no effect on nitrate in the fertilization treatment). However, in the poor soil, earthworm and biochar effects on the availability of nitrate and ammonium are of the same magnitude (+145%). Since in this soil earthwoms but not biochar increased root biomass this suggests that nutrient availability is not the only mechanisms involved, as sugested above.

The increase in the total root biomass in the presence of earhtworms, in the three soil treatments, manifested itself in the three variables measured to describe root architecture. Taken together, root biomass increased in all possible ways in presence of earthworms: total root length, mean root diameter and the number of root ramifications increased in presence of

earthworms. These changes could either be due to an increase in nutrient availability (Alphei *et al.* 1996; Callaham & Hendrix 1998) or to the release of homone-like molecules (Muscolo *et al.* 1998; Nardi *et al.* 2002; Quaggioti *et al.* 2004). However, these earthworm effects were slightly blured by simple effects of biochar and interactions with biochar. Biochar effect on root architecture was overall less clear than earthworm effect. It tended to be of a smaller magnitude than earthworm effect or was even negative in one case (cases of root ramification in the rich soil). Moreover, there were some significant interactions between earthworms and biochar on total root biomass and parameters of root architectures (for exemple biochar increased earthworm effect on total root length in the three soil treatments), while such interactions are very rare for traits describing aerial parts of rice plants. Once again, biochar increased mineral nitrogen availability but did not lead to the same effect as earthworms on roots. All these results support the hypothesis that other factors than nutrient mineralization and availability are involved in earthwom effect on root system. This is also confirmed by the fact that (1) root ramification had the same general level in the three soil treatments, while nutrient availability clearly decreased from the fertilization treatment to the poor soil treatment treatment), (2) root ramification reacted to earthwoms that led to smaller changes in mineral nitrogen availability.

In our study, earthworm and biochar effects on shoot/root ratio strongly varied with the soil type: no effect in the poor soil, increase in the ratio with biochar in the rich soil, decrease in the ratio with earthworm in the fertilization treatment. These effects cannot be directly predicted from effects on the total root biomass: i.e. they result form the interaction between earthworm and biochar effects on the total biomass production and on resource allocation. Shoot/root ratio is generaly though to be primarily by the availability of mineral nutrients (Wilson 1988), which has been affected both by earthworms and biochar. However, the shoot/root pattern we observed is not well correlated with earthworm and biochar effects on mineral nitrogen availability. This suggests that biochar and earthworms might have also directly manipulated resource allocation independently of their effect on nutrient mineralization and retention. This points again at earthworm effect on plant growth through the release of hormone-like molecules that are known to influence the shoot/root ratio (Wilson 1988; Haimi & Einbork 1992; Atiyeh *et al.* 2002).

Effects on grain production

For all soil treatments, the total grain biomass and the grain number tended to exhibit the same tendency as the total biomass. However, neither earthworms nor biochar affected

significantly the mean grain weight in the rich and poor soils. Besides, earthworms both decreased the mean grain weight and the total grain biomass in the fertilization treatment. This effect is not well correlated with the increase in nutrient availability in presence of earthworms. Indeed, earthworms increased the availability of nitrate (independently of biochar) and increased ammonium availability in the biochar treatment. This is again an effect of earthworms on resource allocation. It is correlated with earthworm positive effect on root biomass: in the fertilization treatment earthworms did no influence the total biomass, increased root biomass, and decreased the total grain biomass. It is not clear why rice should decrease its resource allocation to grain in the presence of earthworms in a fertilized soil, unless it is primarily an effect of an increase in the allocation to the root system.

Effects on rice nitrogen content

Earthworms tended to decrease the C/N of all plant parts, even in the fertilization treatment, while biochar had small and less consistent effects on C/N. This effect parallels earthworm effect on mineral nitrogen availability: as usually acknowledged nutrient concentration in plants increase when nutrient availability increase (Lambers 1988; Ingestad & Agren 1992). Such positive effect of earthworms on plant nutrient concentration have already been observed and attributed to their effect on mineralization (Brown *et al.* 2000; Araujo *et al.* 2004). It must however be marked that earthworm effect on leaf C/N is not proportional to their effect on nutrient availability. For example, in the fertilization treatment, the C/N decreased by 47% and mineral nitrogen availability increased by 12%. Meanwhile, the decrease in the C/N of grains and root was relatively much smaller (about 15%). This suggests that rice resource allocation strategy interacts with earthworm effects: a large proportion of nitrogen made available by earthworms is allocated to leaves where it can enhance photosynthesis (Lambers *et al.* 1988b, a). Both an increase in mineral nitrogen absorption (Lehmann *et al.* 2003a; Steiner *et al.* 2007; Steiner *et al.* 2008b) and a decrease in plant C/N have already been observed with the addition of biochar (Lehmann *et al.* 2003b). In our experiment, biochar decreased nitrogen concentration in rice whereas biochar increased both ammonium and nitrate concentration in this soil. Again, effects of treatments on rice did not perfectly parallel their effect on mineral nitrogen availability and biochar and earthworm effects are different. In this case, an explanation could be that earthworms but not biochar increased root biomass in the rich soil, so that earthworms both directly increase nutrient availability and rice capacity to uptake nutrients. This emphasizes again the importance of earthworm effects on plant resource allocation. Biochar negative effect on rice C/N could be

due to the fact that nutrients made available by biochar are not perceived in the same way by plants as nutrients made available by earthworms. For example, cations adsorbed on biochar might be poorly available to plants and thus could not lead to the up-regulation of plant nitrogen concentration (Lambers 1988) and root biomass (Lambers 1988; Wilson 1988).

4.1.6 Conclusion

We found that earthworm and biochar effects highly depend on soil type and we never found a clear positive interaction between earthworms and biochar. Hence, the combination of the two practices could be advised because they have positive additive effects but does not increase further crop production. Due to the fact that our experiment is a short term microcosm experiment, it can however not be excluded that earthworms and biochar could interact synergistically on the long term to increase the availability of mineral nutrients and crop production. Indeed, earthworms increase mineralization, and biochar is likely to increase nutrient retention, so that the two mechanisms could lead to the building up of a much larger nutrient stock (Barot *et al.* 2007b; Boudsocq *et al.* 2009).

The potential benefits of earthworms and biochar highly depend on soil type and on fertilization, which suggests that the use of biochar and earthworms should be more beneficial is some soil types than in other. It has already been recognized that earthworm effect should depend on soil type (Brown *et al.* 2004a) and that the magnitude of earthworm effect does not necessarily decrease with soil fertility (Laossi *et al.* 2009a). As far as we know, while biochar has been said to be beneficial in most tropical soils because they tend to be deprived of nutrients by an intense lixiviation (Lehmann *et al.* 2003c; Glaser & Woods 2004; Asai *et al.* 2009), our experiment is the first one to attempt determining in which soil types biochar should be used. However, as for earthworms, it would be precocious, without further experiment, to predict which soil characteristics determine soil responsiveness to biochar in term of fertility and crop production. Indeed, in our experiment, the "poor" and "rich" soils differed by many other characteristics than their richness in mineral nutrients. To conclude on the interaction between biochar/earthworms and soil type, new experiments using soils with various textures, types of clay or content in organic matter are needed. Finally, the fact that both earthworms and biochar effects tended to disappear with mineral fertilization suggests that biochar and earthworms could be more beneficial instead of mineral fertilization than in combination with mineral fertilization. Other studies have suggested that biochar and mineral

fertilization could increase fertility synergistically (Lehmann *et al.* 2003a; Steiner *et al.* 2007; Steiner *et al.* 2008b). Our experiment does not support this view but this could again be due to the fact that it was not a long term field experiment.

In terms of basic knowledge, our results emphasize the importance of resource allocation. Indeed, it is possible to explain the difference between biochar and earthworm effects using the observation that earthworms, but not biochar, increase the allocation of resources to the root system. We hypothesize that this is due the fact that earthworms and not biochar stimulate some bacteria that release plant growth factors in the soil. Although, other studies support this hypothesis (Muscolo *et al.* 1999; Blouin *et al.* 2006), it should be thoroughly tested, directly assessing the concentration of plant growth factor in the soil of earthworm and biochar experiments and describing more precisely plant physiological responses (Noguera, unpublished result Blouin *et al.* 2005). More generally, effects of soil organisms on plant growth are often interpreted as a consequence of changes in soil properties triggered by these organisms. We show that it cannot be excluded that a significant part of these effects are due to the direct influence of soil organisms on plant resource allocation as already shown within the microbial loop framework (Bonkowski 2004).

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4.1.7 Appendix

Appendix 1: ANOVA table of F value for the effects of biochar (B), earthworms (E) and soils (S) and their interaction on nitrate and ammonium, total biomass, shoot biomass, root biomass, total grain biomass, total root length, mean root diameter, shoot root radio, root ramification, mean grain weight, grain number, grain C/N, root C/N, leaf C/N and total N content.

Table A. ANOVA table of F value for the effects of biochar (B), earthworms (E) and soils (S) and their interaction on nitrate and ammonium. Total df =15. *, p<0.05; **, p<0.01; ***, p<0.001; NS not significant.

	df	Nitrate	Ammonium
B	15	**14.07** ***	**87.87** ***
E	15	**101.72** ***	**98.98** ***
S	15	**558.71** ***	**396.44** ***
E *B	15	0.84 NS	**9.45** *
B *S	15	**9.68****	4.02 NS
E *S	15	**20.91****	**13.75** ***
B*S* E	15	1.85 NS	**10.38** ***
R^2		0.983	0.980

Table B. ANOVA table of F value for the effects of biochar (B), earthworms (E) and soils (S) and their interaction on total biomass, shoot biomass, root biomass, and total grain biomass. Total df = 79 *; **; *** p<0.05, p<0.01, p<0.001, NS not significant.

	df	Total biomass	Shoot Biomass	Root biomass	Total grain biomass
B	1	**91.83****	**63.33** ***	**3.35***	**64.31****
E	1	**128.23****	**152.14** ***	**121.79****	2.55 N.S.
S	3	**128.23****	**878.57** ***	**115.58****	**255.63****
E *B	1	0.17 N.S.	0.78 N.S.	0.99 N.S.	0.11 N.S.
B *S	3	**105.56** ***	**44.19** ***	**3.46** *	**82.11****
E *S	3	**33.57****	**13.3****	2.73 N.S.	**23.21****
B*S* E	3	2.39 N.S.	0.43 N.S.	1.32 N.S.	2.23 N.S.
R^2		0.97	0.97	0.88	0.94

Table C. ANOVA table of F value for the effects of biochar (B), earthworms (E) and soils (S) and their interaction on total root length, mean root diameter, shoot root radio, root ramification, mean grain weight and grain number. Total df = 79 for total root length, mean root diameter, root ramification and grain number. Total df = 78 for shoot root ratio and mean grain weight. *; **; *** p<0.05, p<0.01, p<0.001, NS not significant.

	df	Total root Length	Mean root diameter	Shoot root ratio	Root ramification	Mean grain weight	Grain number
B	1	**5.03***	**5.35** *	**47.12** ***	3.30 NS	0 N.S.	**50.56****
E	1	**83.84** ***	**53.56** ***	**42.6** ***	**54.92****	**7.28** **	**15.96****
S	3	**132.91** ***	**112.39** ***	**355.19** ***	**3.06***	**58.01** ***	**377.07****
E *B	1	**5.57** *	0.95 N.S.	0.01 N.S.	1.00 NS	0.13 N.S.	1.42 N.S.
B *S	3	**6.29** ***	**5.40****	**14.38** ***	**3.17** *	**5.47** **	**38.83** ***
E *S	3	**2.90** *	**4.05** *	**39.67** *	1.89 NS	**5.51** **	**6.00** *
B *S* E	3	1.79 N.S.	1.16 N.S.	**0.22** *	0.39.NS	1.54 N.S.	1.09 N.S.
R^2		0.89	0.87	0.95	0.57	0.77	0.95

Table D. ANOVA table of F value for the effects of biochar (B), earthworms (E) and soils (S) and their interaction on grain C/N, root C/N, leaf C/N and total N content. Total df =47 for grain C/N and total N content. Total df =63 for root C/N and leaf C/N. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NS. not significant.

	df	Grain C/N	Root C/N	Leaf C/N	Total N content
B	1	0.24 N.S.	**65.23 ***	4.67 N.S.	5.68 NS
E	1	**46.75*** **	3.72 N.S.	**135.50 *** **	**186.77*** **
S	3	**7.04*** **	**15.66 *** **	**11.25 *** **	**183.22 *** **
E *B	1	2.34 N.S.	**3.68 ***	0.07 N.S.	1.93 N.S.
B *S	3	2.21 N.S.	**23.95 *** **	**5.65 ** **	**9.82 *** **
E *S	1	**6.01 ***	**6.32 ** **	7.59 N.S.	**5.52 ***
B *S* E	3	0.21 N.S.	**2.21 ***	0.91 N.S.	0.80 N.S.
R^2		0.81	0.81	0.81	0.95

CHAPITRE 4.2:

EFFETS DE L'INTERACTION ENTRE BIOCHAR ET VERS DE TERRE SUR LA CROISSANCE DU RIZ

INFLUENCE DE LA VARIÉTÉ DE RIZ: "CONTRASTED RESPONSIVENESS OF RICE CULTIVARS TO EARTHWORMS AND BIOCHAR"

4.2 Influence de la variété de riz: "Contrasted responsiveness of rice cultivars to earthworms and biochar"

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(Article en première version complète, en attente de soumission)

4.2.1 Introduction

Tropical soils are considered to be particularly vulnerable to fertility losses because of their low capacity to retain organic matter and mineral nutrients (Tiessen *et al.* 1994). This prompts the development of "new" agricultural practices to manage mineral nutrients and organic matter in a more sustainable way while relying less on inputs of fertilizer. Two methods of ecosystem engineering have already been tested with some success. The first one is based on the addition of biochar to the soil and has led to the creation of long-lasting fertility spots by pre-Columbian populations (Glaser 2007). The second one is based on the maintenance of higher earthworm densities while modern agricultural practices drastically deplete earthworm populations (Chan 2001). Earthworms have indeed been shown to increase crop production by direct and indirect effects on plants (Brown *et al.* 1999; Scheu 2003). The two methods are thought to lead to a better nutrient availability (Lehmann *et al.* 2003a) and to involve changes in the soil microbial community (Brown 1995; Pietikäinen & Fritze 2000). Using rice as a model plant, we compared these methods and tested possible interactions between biochar and earthworms in a greenhouse experiment. Besides, crops have to be adapted to their growth conditions and particularly to their soil and common cultivars might not be adapted to new practices. We thus compared the responsiveness to biochar and earthworms of four cultivars of *Oryza sativa* and a cultivar of *Oryza glaberrima.* Results show that there are little interactions between their respective effects. Moreover the different cultivars have very different responsiveness to earthworm, biochar, and the combination of the two. These results have both practical, for the sustainable management of soil fertility, and theoretical implications, for the evolution of aboveground-belowground interactions.

Earthworms are known to influence plant growth through various mechanisms (Brown *et al.* 2004a) such as the increase in mineralization, the production of plant growth substances via the stimulation of microbial activity, biocontrol of pests and parasites, stimulation of symbionts or modification of soil porosity and aggregation. Effects of earthwomrs on plant growth have been extensively studied. They have been shown to be generally positive (Brown *et al.* 1999; Scheu 2003) but it is so far difficult to predict which plant species react positively to earthworms (Laossi *et al.* 2009a) and the respective influence of the different involved mechanisms (Blouin *et al.* 2006). Although, the use of biochar to increase the fertility of tropical soils is probabaly an old practice (Marris 2006), this idea has been rediscovered recently and fewer studies have been published on the effect of biochar on plant growth (but see Glaser *et al.* 2002; Lehmann *et al.* 2003a; Steiner *et al.* 2007) than on the effect of earthworms. Biochar has been schown to imrove various soil characteristis such as its capacity to retain water and nutrients (Glaser *et al.* 2002; Lehmann *et al.* 2003a). It also influences soil microbial community (Pietikäinen & Fritze 2000).

It is relevant to compare the effect of earthworms and biochar on plant growth as well as to study the effect of the effect of the combination of earthworms and biochar. Indeed, earthworms and biochar influence plant growth through mechanisms that are partially the same and that are also interacting. They both modify soil aggregation and the microbial community. Moreover, earthworms increase mineralization while biochar increases the retention of mineral nutrients. This could increase synergeticaly the availability of mineral nutrients to plants as suggest by models of nutrient cycling (Barot *et al.* 2007b). For these reasons earthworms and biochar are likely to interact in the way they influence the growth of plants on the short term. Since, earthworms also directly ingest biochar (Topoliantz & Ponge 2003, 2005) they have also been hypothesized to play an important role in the building up of fertility in biochar enriched soils (Ponge *et al.* 2006). While these issues remain fairly open another one it totally open.

It is known that plant species respond differently to earthworms and they also very likely respond differently to biochar, although this last issue has not been studied systematically yet. It is not known at all how conservative the response is between different genotypes within the same species. Tackling this issue is first essential to use earthworms (Lavelle *et al.* 1989; Lavelle *et al.* 2001) and biochar (Glaser *et al.* 2001) to increase the sustainability of crop production. Indeed, this is only possible if the crop cultivars we cultivate respond positively to increases in earthworms and biochar. We could even imagine

selecting new crop varieties adapted to future agricultural practices based on biochar and earthworms. Tackling this issue could also help, in the long run, to better understand the physiological mechanisms involved in plant response to the presence of biochar and earthworms by comparing the phenotypes of genotypes responding more ore less to these soil features. This issue remains also very open (but see Blouin *et al.* 2005), especially in the case of biochar. Finally, determining how different plant genotypes respond to earthworms is also a way to test for the hypothesis that earthworms represent a selective pressure for plants and that some plant traits have evolved in relation with earthworms. While the relation between plant and microbes is more and more studied from an evolutionary point of view (Lambers *et al.* 2009), the field being probably still in its infancy, this is not the case for plant-soil fauna relations that have hardly been studied from the evolutionary point of view (Barot *et al.* 2007a).

To tackle these issues, a greenhouse microcosm experiment was implemented on five rice varieties (see below the Materials and methods section for details). Three factors were combined in a complete factorial design (see Materials and Methods for details): with/without earthworms, with/without biochar and with/without fertilizer. This last treatment aims at mimicking agricultural practices. It is particularly relevant within the framework of the study because the availability of nutrients may change plant responsiveness to earthworms and biochar. On the one hand, fertilizer could decrease the impact of earthworms and biochar on plant growth if they mainly act via an increase in mineral nutrient availability (Blouin *et al.* 2006; Laossi *et al.* 2009a). On the other hand, biochar and fertilization could interact in a synergetic way to build up fertility (Lehmann *et al.* 2003a), and thus fertilization could increase plant responsiveness to biochar.

4.2.2 Materials and methods

Experimental design

The experiment was conducted at CIAT (International Center for Tropical Agriculture) in Cali, Colombia. Plants were submitted to the four possible combinations of two factors: with and without earthworm (respectively noted E, NE) and with and without biochar (respectively B, NB). All treatments combinations were implemented for the African rice (*Oryza glaberrima,* IRGC 103544) and four Asian rice cultivars (*Oryza sativa*) and: *cv. Line 30* (accession CIRAD 409), *Azucena* (accession IR64), *Nipponbare* (accession IRGC

12731) and "Donde lo tiren". *O. glaberrima* has been selected and cultivated in parts of West Africa for more than 3500 years. It has developed adaptive mechanisms for resisting major biotic and abiotic stresses. *Line* 30 has been developped conventionnaly by hibridation. It is well adaptated to colombian savannas and is widely used since the 90s. *Azucena* is a semidwarf type derived from extensive intercrossing of improved lines, and it has good yield potential and resistance to numerous biotic stresses. It is the most widely grown rice variety in the tropics. *Nipponbare* is a Japanese cultivar used as model rice genotype (it has been sequenced). It is the outcome of successive selection on Japonica varieties and is characterized by a good sucker growth. *Donde lo tiren* is a Colombian local rustic landrace.

For each combination of treatments (E x B x rice cultivar), two fertilization treatments were implemented: without or with mineral fertilization. Five replicates were implemented for each treatment, resulting in 200 microcosms. Rice was grown in a greenhouse for four months.

Containers (microcosms) consisted of PVC pots. They were filled with 1500 g (+/- 50g) of sieved (2 mm) dry soil. Microcosms were arranged in a completely randomized design. The soil was collected from one long term field experiments that aimed at comparing coffee production with and without the addition of biochar that was established in 2004, in the Andean hillsides of the Cauca Department (Pescador, 2° 48'N 76° 33' W). Soil was collected in the control treatments of these experiments for our microcosm NB treatment, and from their biochar treatments for our microcosm B treatment. The soil of this treatment contained 25 g of biochar per dry kg of soil.

The "Pescador" soil is a volcanic-ash soil, an inceptisol (USDA, 1998). The soil is moderately acid (pH (H_2O) = 5.1). It is relatively rich in organic matter MO (11.5 %), and mineral nitrogen (12.9 mg NH_4^+-N kg^{-1}, 27 mg NO_3^--N kg^{-1}). The CEC is relatively high (6.0 cmol kg^{-1}). Texture is dominated by clay (24.06% sand, 27.56% silt, and 48.38% clay). The soil bulk density is 0.8 g cm^{-3}. The low fertility treatment consists in an application of a N, P, K and S fertilizer (26.09 g N, 37.5 g P, 28.85 g K and 3.49 g S per kg of dry soil). The fertilizer was placed at the soil surface at the beginning of the experiment and corresponds to 46 kg N; 20 kg P; 52 kg K and 7.5 kg S per hectare (considering a 10 cm deep soil layer).

We used for each microcosm five adults (initial fresh weight 5 +/0.5 g) of *Pontoscolex corethrurus* (Glossoscolecidae). This earthworm is a peregrine species that has spread in all the tropics. Three days after introducing earthworms, 5 rice seeds: were sown. Two weeks later, a single plant per microcosm was kept (the other seedlings were removed). Microcosms

were regularly weeded during the experiment and wer maintained at 80% of the soil field capacity (checked through regular weighing of the pots).

Measurements

After sixteen weeks, plants were harvested and separated into grains, leaves, and stems. Roots were collected by wet sieving. The vegetal biomasses were dried in an oven at 40°C for 2 days. Grains were counted. Subsamples of each plant material were analyzed for total carbon, total nitrogen and using a ThermoFinnigan Flash EA 1112 elemental analyzer (ThermoFinnigan, Milan, Italy).

Statistical analyses

We base the analysis of our results on the effect sizes of the different treatments (Nakagawa & Cuthill 2007). This approach allows focussing on the magnitude of the effects of the different treatments comparing the magnitude of these effects, in comparison to the control treatment, in different cases: here the rice variety and the fertilization treatment. This also helps displaying results in a more synthetic way. We used a standardized statistics, Cohen's *d* *(Cohen 1988)*, which the difference between the treatment and control mean values divided by the pooled standard deviation. *d* was displayed together with its standard deviation which can be easily computed using the sample size and the estimated *d* value (Hedges 1981). Effect sizes were calculated separately for each rice variety and for each fertilization treatment (with and without).

To complete this approach, histograms of the raw means of the different variables are displayed for all treatments in the Supplementary Material together with ANOVA tables for the full statistical models testing for the earthworm, biochar, fertilization and cultivar effects as well as for all interactions between these factors. This should convince readers not familiar with the interpretation of effect sizes that earthworm and biochar effects on rice growth do change with rice cultivars and fertilization (numerous significant interactions between on the one hand earthworm and biochar treatment and on the other hand rice variety and fertilization.

4.2.3 Results

The full statistical model (see Supplementary material) shows that for most output variables there is significant interaction between rice variety and both biochar and earthworms. This proves that rice varieties have different responsiveness to biochar,

earthworms and the combination of biochar and earthworms. Hereafter, we base the description of our results on the calculation of effect sizes (ES, see Materials and methods) that denote the strength of the effects relatively to the control treatment (within a fertilization treatment). In all cases, i.e. all varieties and with or without fertilization, the treatments increase the total rice biomass (Fig .1, positive ES). This total biomass tends to respond more to earthworms and biochar without fertilization than with fertilization .With fertilization, the strongest effect is obtained with both biochar and earthworms in *Nipponbare* and *O. glaberrima* (ES about 5). Without fertilization, the strongest effect is obtained with earthworms in *Azucena* and *Donde lo tiren* (ES about 7) and with both biochar and earthworms in *Donde lo tiren (*ES about 9*)*. Effects of earthworms and biochar on the shoot/root ratio are different with and without fertilization. Earthworms increase the shoot/root but in a much stronger way without fertilization. Biochar tends to decreases the shoot/root ratio but only without fertilization. The strength of the effect depends on the rice variety and the on the interaction between biochar and earthworms! With fertilization the strongest increase is obtained with earthworms in *O. glaberrima* (ES about 4). Without fertilization, the strongest increase is observed with the combination of earthworms and biochar in *O. glaberrima*, and the strongest decrease is obtained with biochar in *Donde lo tiren* (ES about -1). Overall, the pattern of responsiveness of the total grain biomass is the same in the two fertilization treatments: *Nipponbare* has the highest responsiveness to biochar (ES between 2 and 3) and earthworms (ES between 1 and 2) and *line 30* has the highest responsiveness to the combination of biochar and earthworms (ES about 3). In some varieties, biochar or earthworms have a negative impact on the production of grain biomass (negative ES). This pattern is different form the one observed for the total biomass, which, together with results on the shoot/root ratio, the leaf C/N and the number of grains (see Supplementary material for these last two variables), shows that earthworms and biochar influence rice resource allocation in terms of biomass and nitrogen, and that these effects on resource allocation differ between rice varieties.

4.2.4 Discussion

The complete interpretation, in terms of mechanisms, of the effects of biochar and earthworms on rice biomass accumulation and resource allocation goes beyond the objective of the present article. Another experiment (Noguera's PhD) focussing on one rice variety (*line 30*), but comparing different soil treatments, suggests that both earthworms and biochar first

act through their positive effect on the availability of mineral nutrients but that earthworm effect cannot be fully understood without assuming that they lead to the release of plant growth factors in the soil through the stimulation of particular bacteria groups (Muscolo *et al.* 1999; Quaggioti *et al.* 2004). Here, the key point is that the pattern of response of rice varieties is complex: the responsiveness to biochar, earthworms and the combination of the two is generally different, and is often different with and without fertilization. Besides, this responsiveness is very different depending on the examined variable. As it is so far not possible to predict the responsiveness of different plant species to earthworms (Laossi *et al.* 2009b) and biochar it seems impossible to predict the responsiveness of different rice varieties. This is not a surprise due to the complexity and abundance of underlying mechanisms and, in the case, of biochar, due to the fact that very few studies have studied in details its effects on plant growth. In fact, small differences between genotypes in the root foraging strategies, root exudation, general resource allocation strategy, and physiological regulations are probably sufficient to trigger important changes in their responsiveness to earthworms and biochar. Elements of explanation could probably be found examining, for example, what is known about the root architecture of rice varieties. However, collecting new data on the physiological response of rice cultivars to earthworms and biochar would probably be required to predict their responsiveness in term of yield (see for example Blouin *et al.* 2005).

Crop breeding has mostly focussed on improving cultivar resistance to pathogens and parasites, their capacity to resist to drought, and their capability to benefit from mineral fertilization (Hoisington *et al.* 1999; Pennisi 2008; Witcombe *et al.* 2008). Our results are thus rather original, but they may also appear useless to agronomists and plant breeders. Why focussing on responsiveness to biochar and earthworms? For biochar, the answer is very clear: if we want to develop (or redevelop them) new agricultural practices, we also need to develop the suitable cultivars that highly benefit from these practices. For earthworms, the answer is threefold. First, modern agricultural practices such as tillage and negative consequences of these practices on soil organic matter content have often a negative impact on earthworms (Fragoso *et al.* 1997). Conversely, alterative agricultural practices aiming at more sustainability often have positive impact on soil fauna and earthworms (Mäder *et al.* 2002). Such practices would benefit form earthworm responsive cultivars. Second, practices are proposed to directly increase earthworm biomass to restore soil fertility (Lavelle *et al.* 1989). Such practices would particularly benefit from the use of responsive cultivars. Third,

soil organisms are more and more thought to play an essential role in the sustainability of soil capacity to sustain vegetal production because it is involved in positive feedback loops between soil properties and plant production (Lavelle *et al.* 2001; Wardle *et al.* 2004; Bardgett *et al.* 2005; Lavelle *et al.* 2006); It might thus be possible to foster further these positive effects on sustainability through the use of responsive cultivars. Such an approach is currently in development for mycorrhizae (Sawers *et al.* 2008). Mycorrhizae are an obvious place to start because of their symbiotic association wit plants, however most soil organisms interact directly or indirectly with plants (Wardle *et al.* 2004) so that the approach might be applied to many other organisms such as earthworms. This would be a way to green the green revolution (Tilman 1998) and to favour the ecological intensification of crop production (Matson *et al.* 1997; Cassman 1999).

Other results point at the fact that cultivars respond differently to soil organisms such as mycorrhizae (Zhu *et al.* 2001) or protozoa (Somasundaram *et al.* 2008). This suggests that our results can be generalized to all influential groups of soil organisms. Another issue is then to find genotypes having high responsiveness to the desired organisms. It is well possible that modern cultivars have lost some of the traits that allow them to interact efficiently with soil organisms. Indeed agricultural practices tend to be unfavourable to many groups of soil organisms and many cultivars have been developed to growth efficiently, only when provided with an abundant mineral fertilization. Consequently, subtitle mechanisms allowing plants to access mineral nutrients through complex rhizospheric interactions (involving interactions with soil organisms) (Bonkowski 2004)might have been selected against or might have been stochastically lost during the selection of high yielding cultivars that have started thousands years ago. A solution might thus be to find lost traits in local landraces or non-domesticated ancestors (McCouch 2004). Our results support these views in the sense that *Donde lo tiren* is the more rustic rice variety of our experiment and responds well to earthworms in term of total biomass without mineral fertilization. However, due to interactions with the resource allocation strategy, *Donde lo tiren* responds slightly negatively (negative ES) to earthworms in terms of grain biomass while modern cultivars such as *Line 30* and *Nipponbare* increase very significantly their grain biomass in presence of earthworms (ES higher than 2). This is probably due to the fact that modern cultivars have been selected to allocate more resources to grains and shows that the naïve view that good genes are always to be found in old or local cultivars does not hold.

We have shown that earthworms and biochar influence rice growth and resource allocation in interaction with rice genotype. We also know that earthworms and biochar act through various mechanisms involving the spatial and temporal patterns in the availability of mineral nutrients, root architecture and dynamics, interactions with microorganisms...etc. All these mechanisms are essential to plant functioning and the sustainability of crop yield. This suggests that determining precisely how plant traits influence their response to earthworms and biochar and unravelling the underlying molecular mechanisms is likely to throw new lights on soil-plant interactions and some fundamental plant physiological mechanisms.

Finally, it is known that plant species have various responsivenesses to earthworms (Wurst *et al.* 2005; Milcu *et al.* 2006; Wurst *et al.* 2008; Laossi *et al.* 2009b) and we have shown here that plant genotypes, within a species, might also differ by their responsiveness to earthworms. This strongly suggests that earthworms could represent a selection pressure for plants. To do so, earthworms do not only need to influence the biomass production of different genotypes in contrasted ways; they also need to influence differently their fitness. Our results on the number of grains produced (see Supplementary material) support this view. Other studies have shown that, earthworms have contrasted effects on the demography of plant species via various mechanisms (Decaens *et al.* 2003; Laossi *et al.* 2009b) that could also lead to differences between the fitness of different genotypes. Of course, genetic differences between rice varieties have arisen through artificial selection and might not reflect quantitatively and qualitatively differences arising naturally between the genotypes of conspecific plant individuals within a grassland. The effect of such differences on responsiveness to earthworms should thus be investigated in the future. Nevertheless, the evolution of the interactions between plants and belowground organisms has mostly been studied from the point of view of symbiotic relations (Denison 2000; Karst *et al.* 2008). Our result suggest that it is relevant to extend this approach to non-symbiotic soil organisms that influence plants, as earthworms, through their activity of ecosystem engineers (Jones *et al.* 1994), modifications of the community of microorganisms, effects on soil food web and, the spatial and temporal patterns of mineral nutrient release. This would help developing a general evolutionary framework that is currently missing in soil ecology (Crawford *et al.* 2005; Barot *et al.* 2007a).

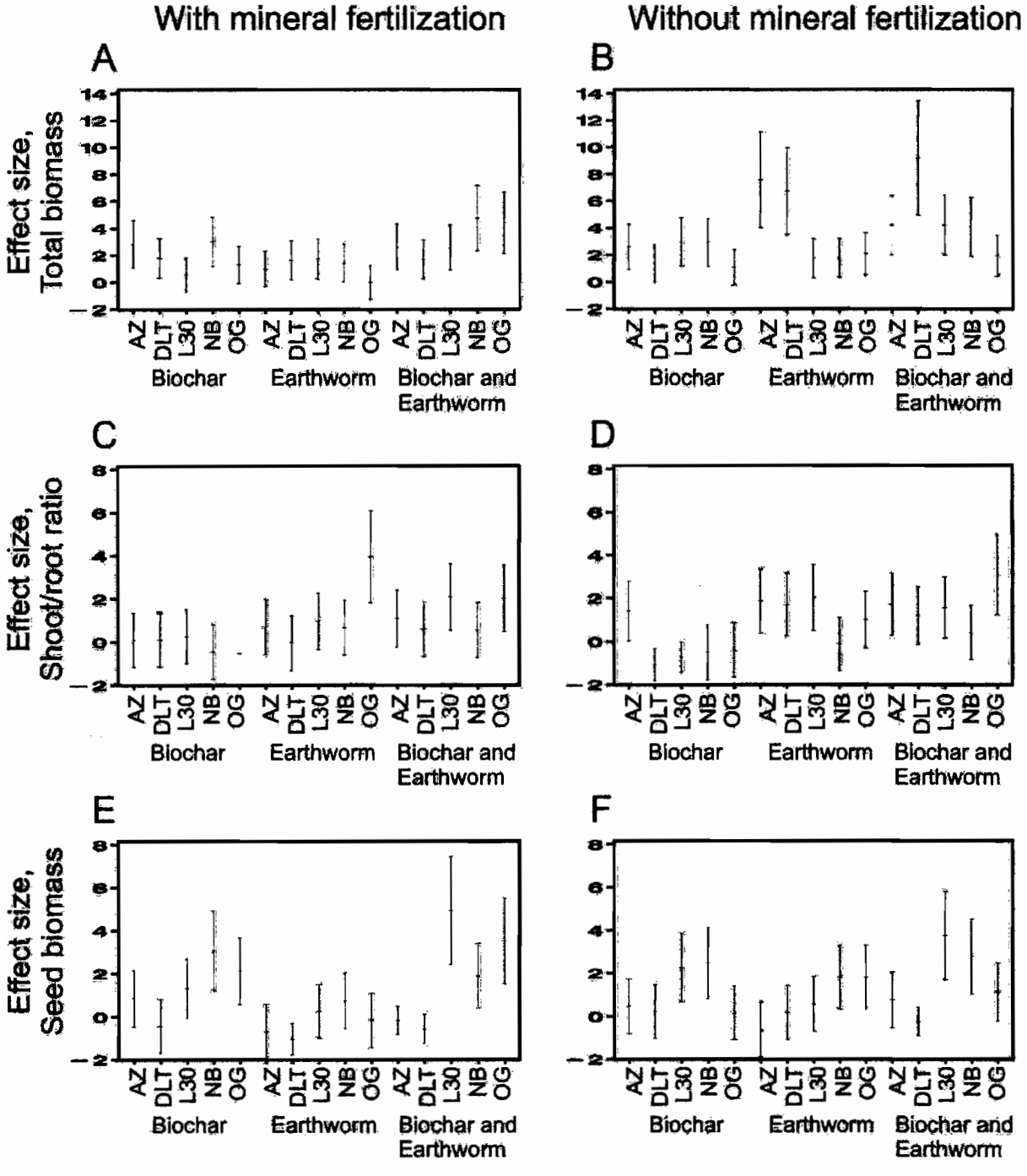


Fig. 1 Effect sizes of the different treatments (biochar, earthworm, and combination of biochar and earthworm) on the total biomass (A-B), the shoot/root ratio (C-D) and the total biomass of grains (E-F). In each case effect sizes are displayed for the mineral fertilization treatment (left-hand column of panels) and the non-fertilized treatment (right-hand column of panels), and for each rice cultivar (AZ, Azucena; DLT, Donde lo tiren; L30, linea 30; NB, nippon bare) and the African rice (OG, *Oryza glaberrima*).

4.2.5 Supplementary material

1 ANOVA (SM Table 1) and raw data (SM Fig. 1-5)

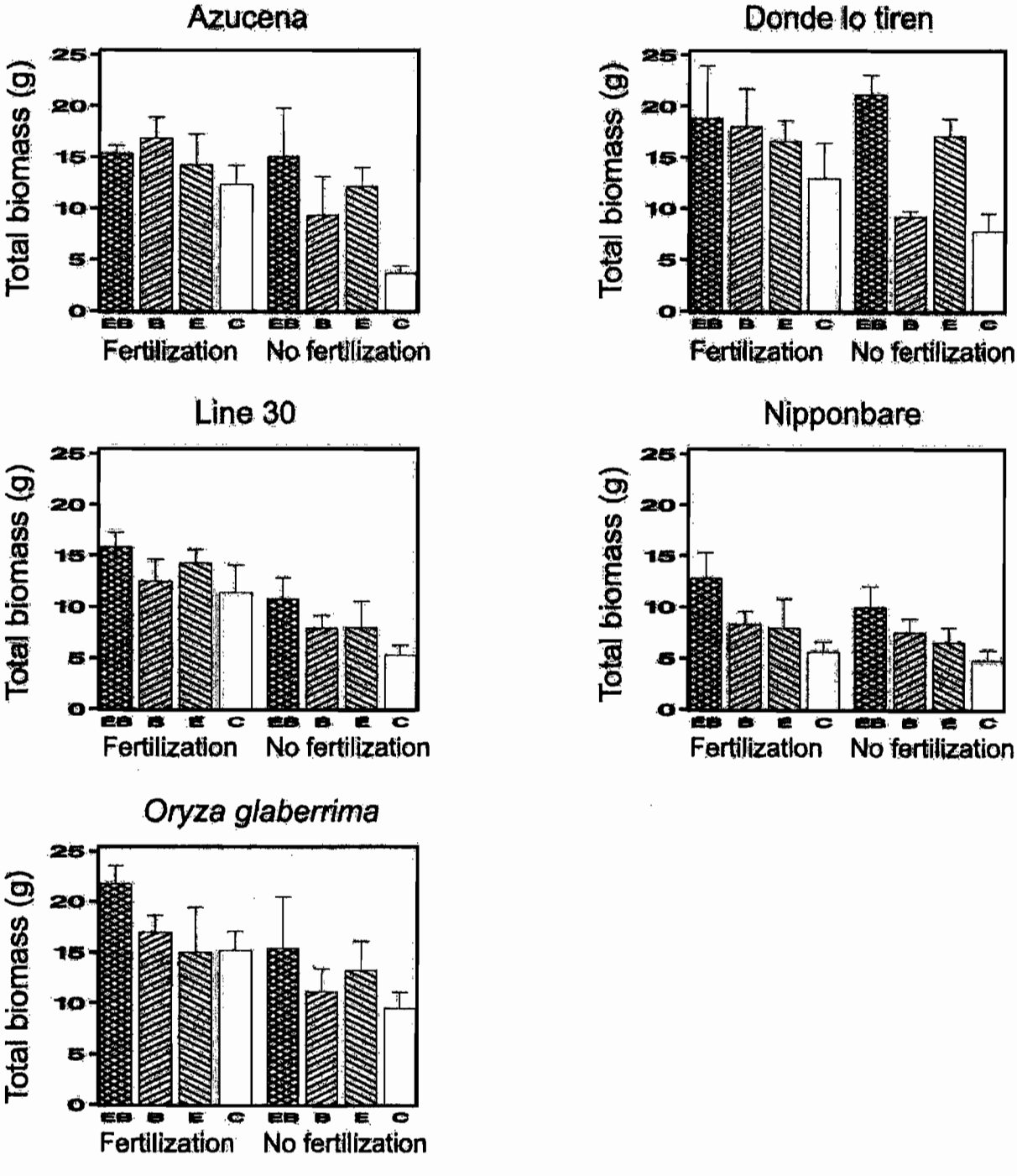
2 Effect size for the leaf C/N and the grain number (SM Fig. 6)

SM Table 1: ANOVA table for the full statistical model with the four factors and all interactions. F values are displayed. The total degree of freedom is199 for all variables but the leaf C/N. In this last case, it is 116 (the five repetitions were not analysed). *, P<0.05 ; **, P<0.01 ; ***, P<0.001. V, rice variety; B, biochar; E, earthworms; F, fertilization.

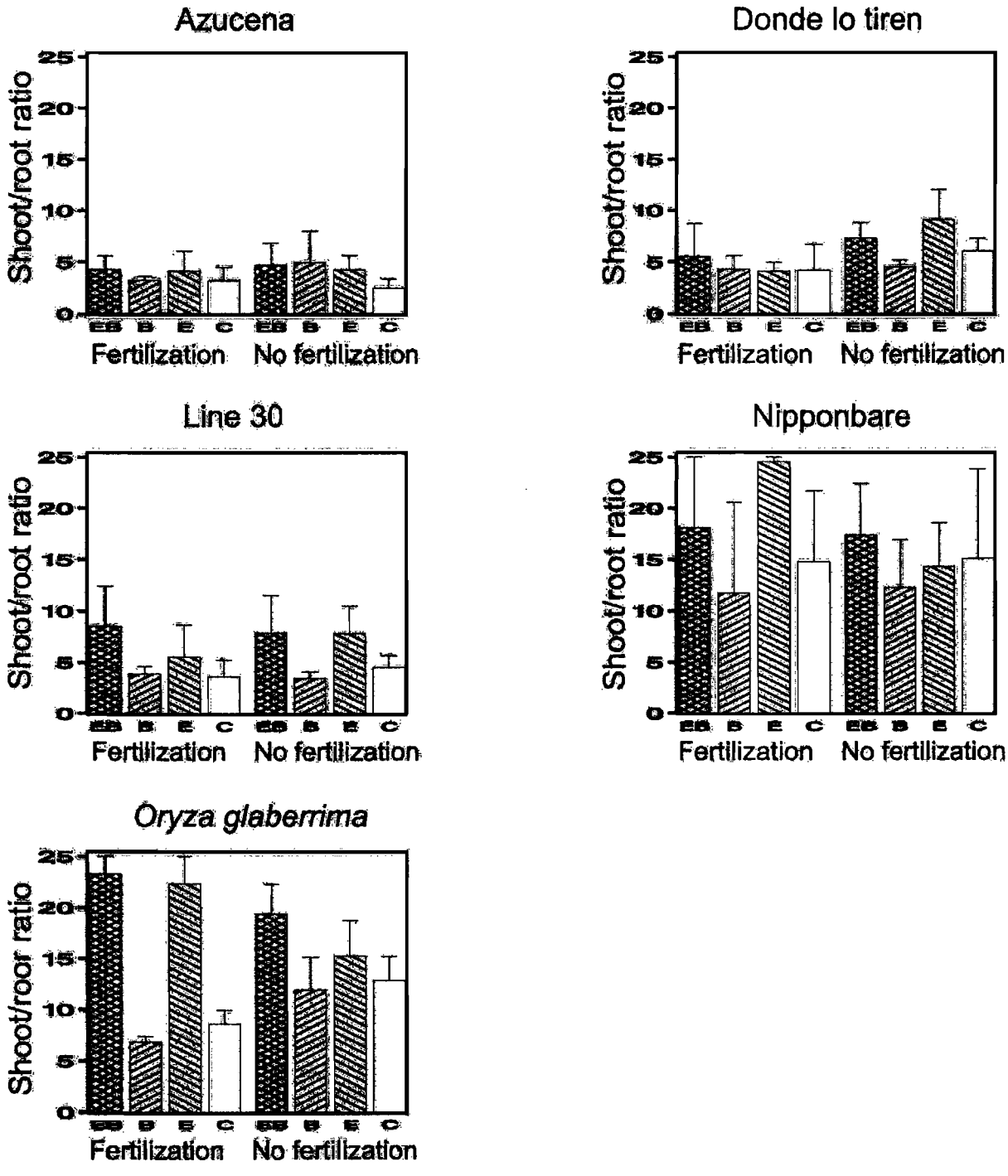
	df	Total biomass	Shoot/root ratio	Total grain biomass	Leaf C/N	Grain number
V	4	**89.1*** **	**60.77*** **	**191.1*** **	**74.7*** **	**117.0*** **
B	1	**114.9*** **	0.1	**46.8*** **	**20.9*** **	**36.0*** **
E	1	**177.9*** **	**41.2*** **	**16.9*** **	**8.34** **	**33.0*** **
F	1	**184.0*** **	0.0	**53.3*** **	**7.0** **	**56.0*** **
VxB	4	0.9	0.8	**7.2*** **	**22.7*** **	**5.1*** **
VxE	4	**5.6*** **	**5.9*** **	**6.0*** **	**6.2*** **	**10.7*** **
VxF	4	**7.0*** **	1.3	**11.1*** **	**3.3***	**11.0*** **
BxF	1	0.1	0.3	1.6	0.13	0.8
BxE	1	0.2	1.0	2.9	0.62	0.8
ExF	1	**29.2*** **	**4.0***	3.2	0.0	2.9
VxBxE	4	2.9*	0.5	1.1	1.4	0.8
BxExF	1	0.0	0.2	0.1	1.95	0.8
VxBxF	4	1.7	1.1	1.0	2.2	**2.75***
VxExF	4	**11.7*** **	**3.1***	1.4	0.8	2.2
VxBxExF	4	2.0	0.7	0.3	1.4	1.8
R^2		0.86	0.68	0.86	0.86	0.82

SM Fig. 1-5 Mean values for the total biomass (SM Fig. 1), the shoot/root ratio (SM Fig. 2), the total grain biomass (SM Fig. 3), the leaf C/N (SM Fig. 4), and the number of grains (SM Fig. 5). Means are displayed for all combinations of the four factors: biochar, earthworm, fertilization and rice variety. Error bars denote standard deviations.

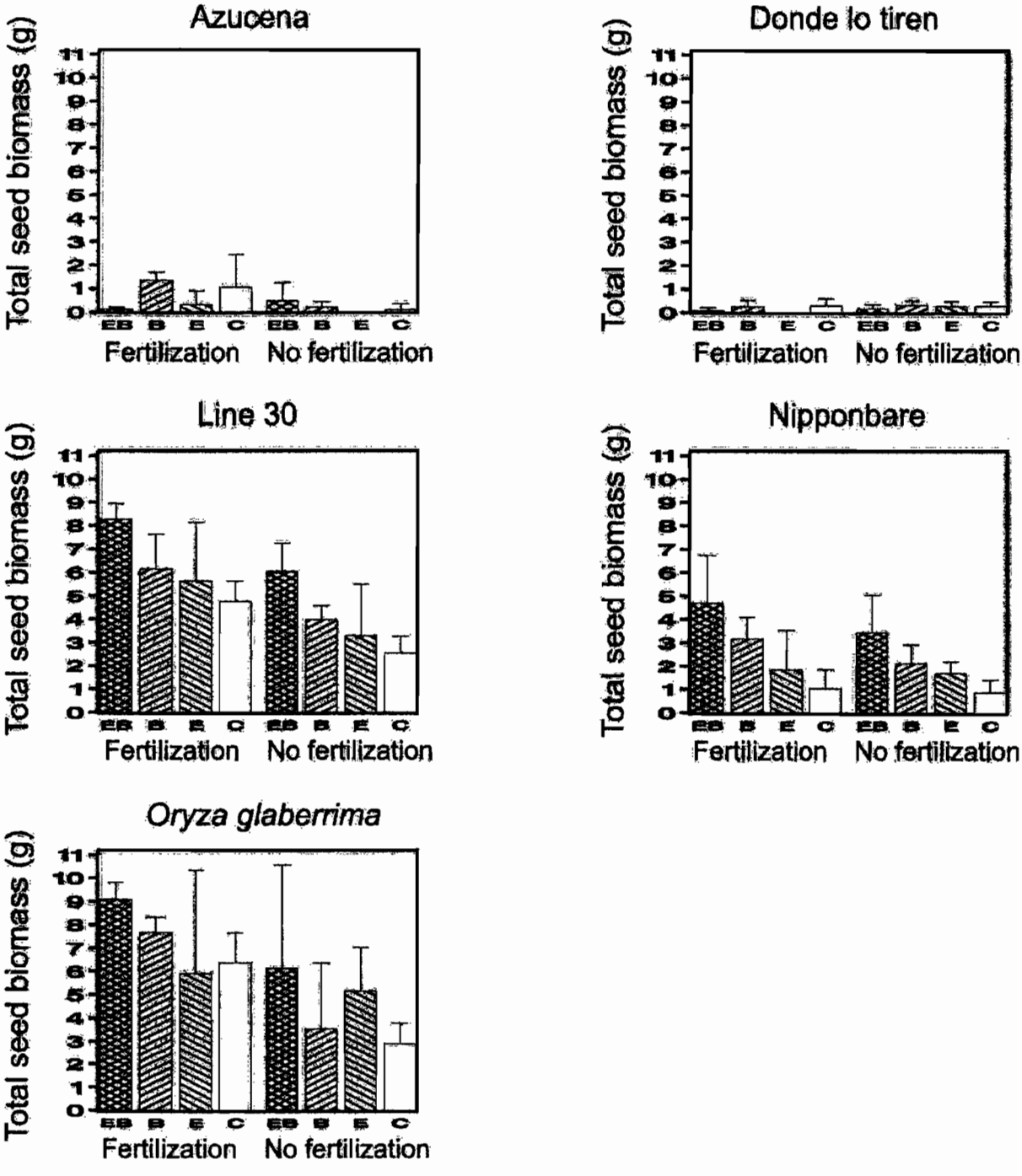
SM Fig. 1



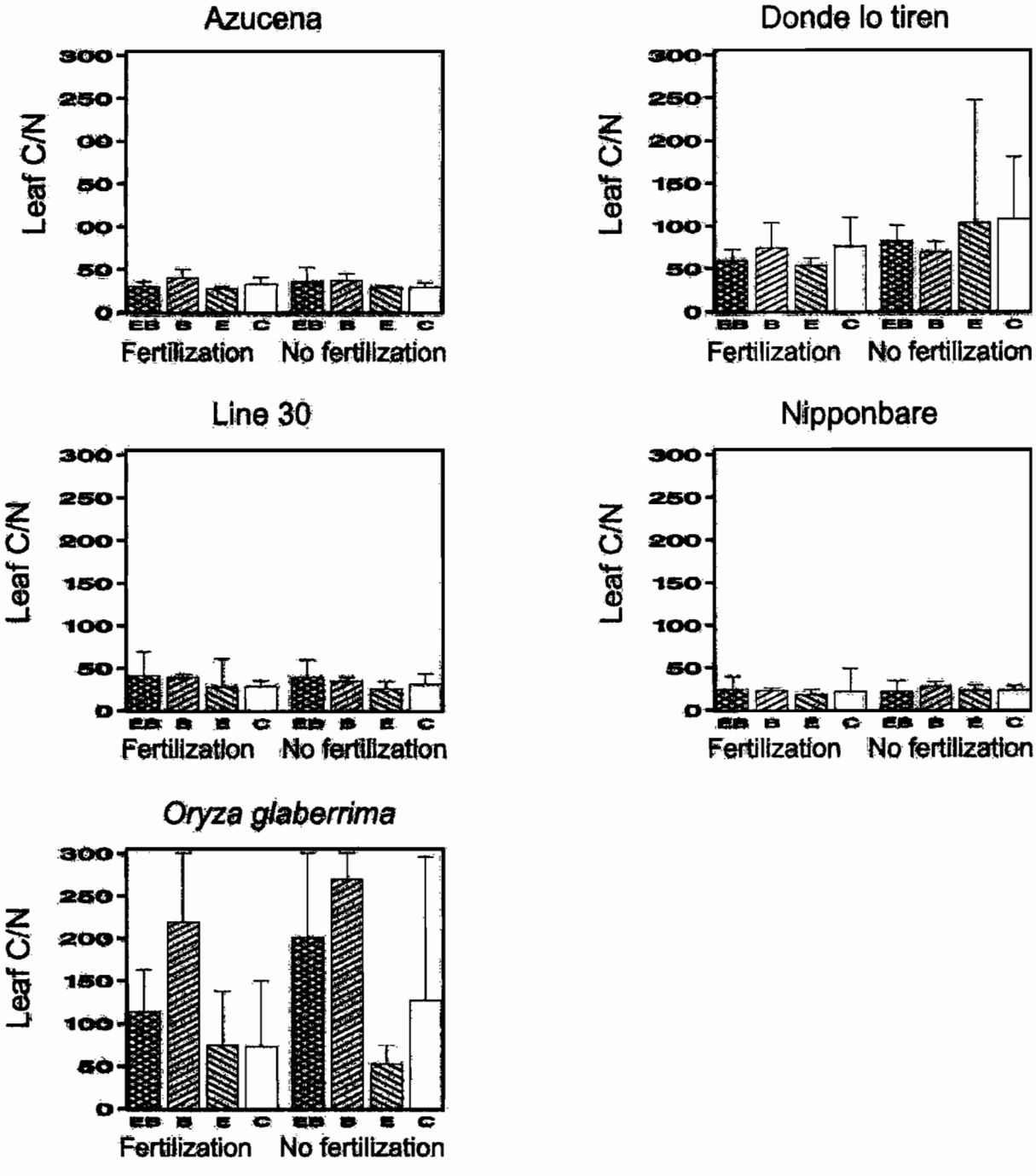
SM Fig. 2


Azucena
Donde lo tiren
Line 30
Nipponbare
Oryza glaberrima
Shoot/root ratio
Shoot/roor ratio
0
5
10
15
20
25
EB B E C EB B E C
Fertilization
No fertilization

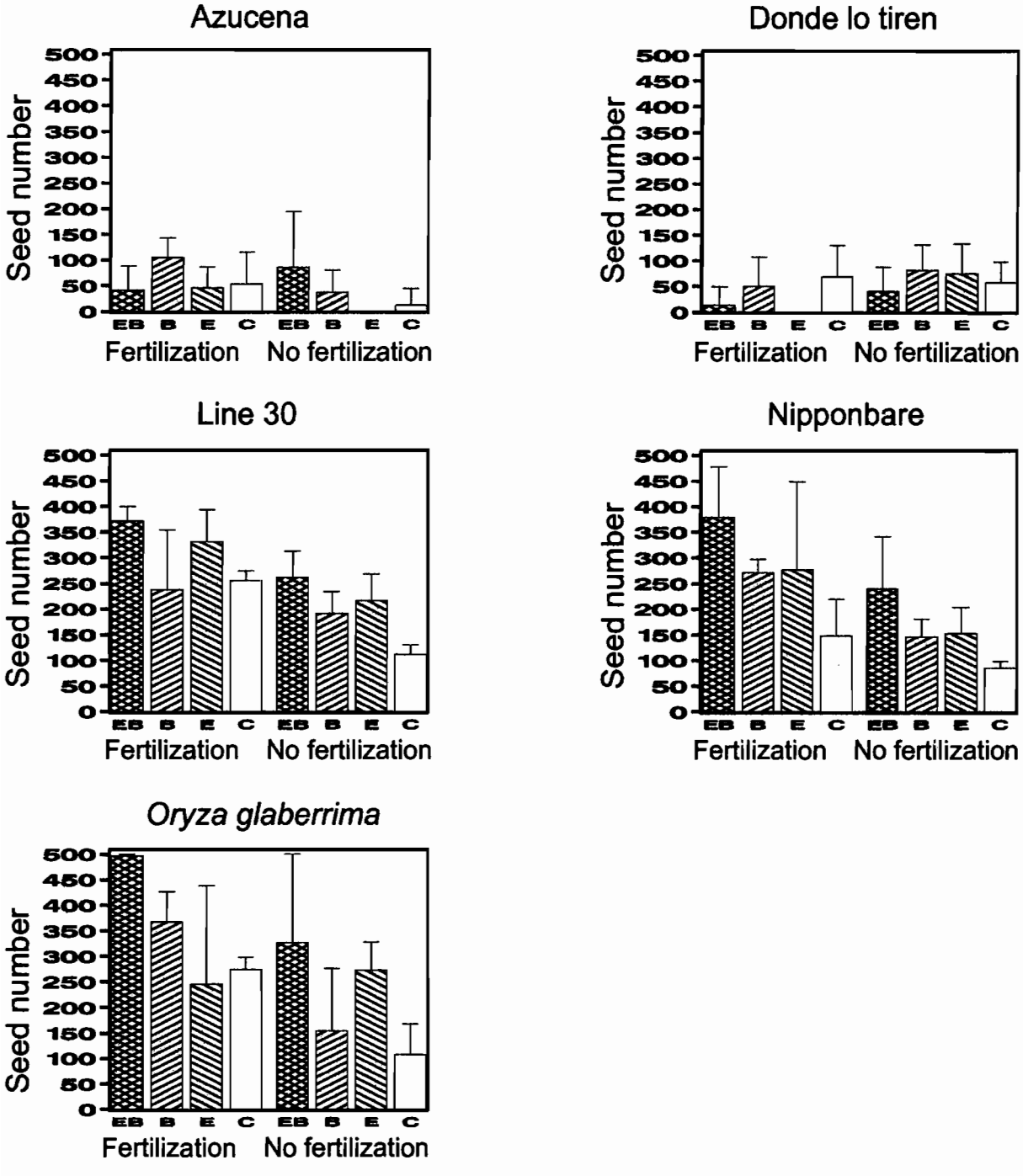
SM Fig. 3


Azucena
Donde lo tiren
Line 30
Nipponbare
Oryza glaberrima
Total seed biomass (g)
0 1 2 3 4 5 6 7 8 9 10 11
EB B E C EB B E C
Fertilization
No fertilization

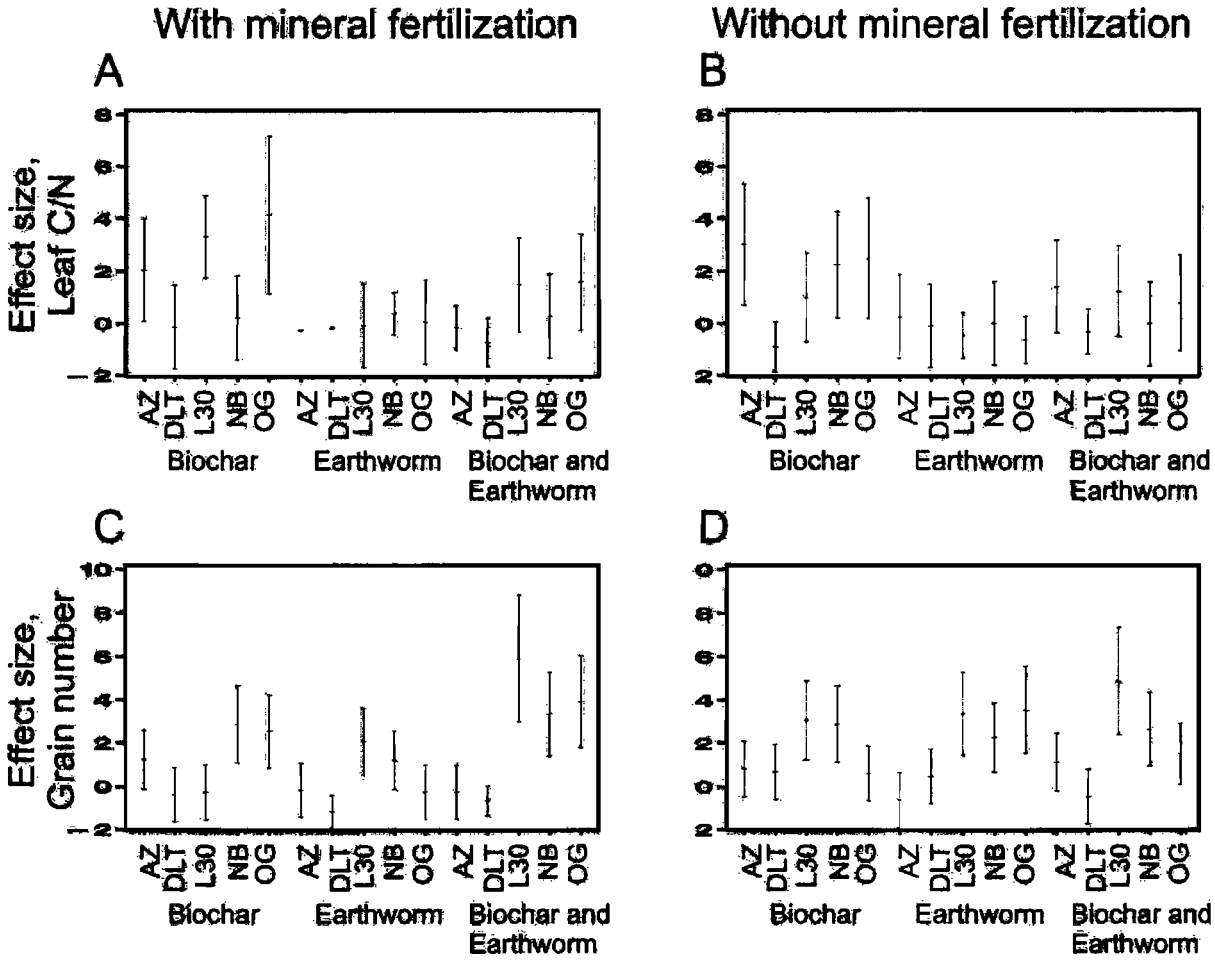
SM Fig.4


Azucena
Donde lo tiren
Line 30
Nipponbare
Oryza glaberrima
Leaf C/N
300
250
200
150
100
50
0
EB B E C EB B E C
Fertilization
No fertilization

SM Fig. 5


Azucena
Donde lo tiren
Line 30
Nipponbare
Oryza glaberrima
Seed number
0
50
100
150
200
250
300
350
400
450
500
EB B E C EB B E C
Fertilization
No fertilization

SM Fig. 6 Effect sizes of the biochar, earthworm, and combination of biochar and earthworm treatments on the leaf C/N (A-B) and the grain number (C-D). In each case effect sizes are displayed for the mineral fertilization treatment (left-hand column of panels) and the non-fertilized treatment (right-hand column of panels), and for each rice cultivar (AZ, Azucena; DLT, Donde lo tiren; L30, linea 30; NB, nippon bare) and the African rice (OG, *Oryza glaberrima*).



CHAPITRE 5:

EFFETS DE L'INTERACTION ENTRE BIOCHAR ET VERS DE TERRE SUR LA PHYSIOLOGIE DU RIZ:

"BIOCHAR BUT NOT EARTHWORMS ENHANCES RICE GROWTH THROUGH INCREASED PROTEIN TURNOVER"

5 Effets de l'interaction entre biochar et vers de terre sur la physiologie du riz: "Biochar but not earthworms enhances rice growth through increased protein turnover"

Diana Noguera, Sébastien Barot, Kam-Rigne Laossi, Juan Cardoso, Patrick Lavelle, Maria H. Cruz de Carvalho

(Article soumis à Environmental and Experimental Botany)

5.1 Abstract

Biochar and earthworms influence positively plant growth through mechanisms that are partially the same: they both change soil structure and soil microbial community and influence nutrient cycling. To compare the effect of biochar and earthworms on plant growth and to investigate the possible interactions between both, a greenhouse experiment on rice was implemented. In addition to classic macroscopic variables we also monitored some leaf-level cellular processes involving protein turnover. Both biochar and earthworms had a significant effect on shoot biomass (+163% and +98%, respectively). However, biochar had a higher effect on the number of leaves (+87%) and earthworms on leaf area (+89%). Biochar also had a significant effect on the leaf dynamics since the number of dead leaves was also superior. At the cellular level biochar enhanced protein catabolism by a significative increase of leaf proteolytic activities. This could be related to 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 of the cysteine protease gene *OsCatB* (+257%). Furthermore, biochar also enhanced the expression level of the two genes, tested for 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. Therefore our data gives evidence that biochar increases rice biomass production by increasing protein turnover (catabolism and anabolism). On the other hand, earthworms did not have such an effect on protein turnover and when used in combination, they attenuated the biochar effect. We finally

draw an analogy between the shifts triggered respectively by biochar and earthworms in rice protein metabolism and *r* and *K*-selected species, as described by the ecological theory. We suggest that importing this theory originally designed at the species level could be useful to interpret plant physiology.

Keywords: Earthworms, *terra preta*, biochar, nitrogen, N turnover.

5.2 Introduction

Many soils of the lowland humid tropics are thought to be too infertile to support sustainable agriculture. One of the major problems of sustainable agriculture in the humid tropics is the rapid decomposition of organic matter (Zech 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, and the agriculture is 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 a priority. The use of more stable organic matter could thus help increase the sustainability of soil fertility. In this context, biochar addition to soils seems to be a promising alternative to transfer of more easily decomposable organic matter (Zech 1990; Glaser *et al.* 1998; Fearnside *et al.* 2001). Indeed, the existence of anthropogenic biochar-enriched dark soil (*terra preta de indio*) and the fact that they have kept a high fertility for hundreds of years supports this idea. Apart from high SOM contents, the most striking feature of biochar is its high nutrient content (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. This suggests that creating modern *terra preta* could be a way to increase tropical soil fertility (Marris 2006).

Maintaining high biomasses of earthworms could be another sustainable way to increase tropical soil fertility (Lavelle *et al.* 2001). They are also known to affect plant growth, generally positively, via five main mechanisms (Scheu 2003; Brown *et al.* 2004a): (1) an increased mineralization of soil organic matter (2) the production of plant 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.

Biochar and earthworms apparently influence plant growth through mechanisms that are, at least to some extent, the same: they both change soil structure and soil microbial community (Pietikäinen & Fritze 2000; Brown *et al.* 2004a) and influence nutrient cycling. While earthworms increase organic matter mineralization on the short term (Scheu 2003; Brown *et al.* 2004a), biochar increases the retention of mineral nutrients (Lehmann & Rondon 2006) which decreases lixiviation and is likely to increase nutrient availability on the long term (Lehmann *et al.* 2003a; Lehmann *et al.* 2003b).

To compare the effect of biochar and earthworms on plant growth and to investigate the possible interactions between both, a greenhouse experiment on rice was implemented. Generally, ecological studies on plant responses to a particular soil treatment have focused on either root-level responses (Gregory 2006) or community-level changes , and very few studies have included measurements of leaf-level physiology (Day & Detling 1990b, a; Jaramillo & Detling 1992a, b; Peek 2003; Blouin *et al.* 2005) and up to now, the molecular processes underlying the observed changes in plant growth and morphology has only once been addressed (Blouin *et al.* 2005). 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 earthworm and biochar influence plant growth. To tackle these issues, in addition of macroscopic variables we monitored some leaf-level cellular processes involving protein turnover.

Plant scientists have long recognized protein turnover as a fundamental component in plant development. Research has however, traditionally focused only 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 likely lead to different regulations of protein synthesis and degradation.

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 classes of proteases: aspartic acid, serine, cysteine and metalloproteases. Since proteases can cleave more or less any available protein, they are present in specific cellular

compartments, namely in acidic vacuoles (Callis 1995). Beside this, 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 & Ohira 1982). Approximately 80 % of total leaf N is invested in chloroplasts (Makino & 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 (Kumar *et al.* 2002; Makino 2003; Makino *et al.* 2003). It comprises eight small subunits (*SSUs*) and eight large subunits (*LSUs*), which are products of the nuclear *rbcS* genes 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 accounts 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, is closely related to both C and N economy in plants (Imai *et al.* 2005).

In this context, our study aimed at testing the following hypotheses: (1) Since biochar and earthworms influence plant growth at least through an increase in mineral nutrient availability, they should influence nitrogen metabolism, namely protein turnover. (2) Since biochar and earthworms influence plant growth through partially different mechanisms, they should affect plant physiology differently at the 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 with the underlying processes related to protein turnover operating at the cellular levels.

5.3 Materials and methods

Substrate preparation

The soil used in this work 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). The Pescador soil is a volcanic-ash soil, an inceptisol, in the USDA classification system

(USDA, 1998). This soil is called hereafter the rich soil (R) because of its higher agronomic quality. The soil is moderately acid (pH = 5.1). It is relatively rich in organic matter MO (11.5 %), and mineral nitrogen (12.9 mg NH_4^+-N kg^{-1}, 27 mg NO_3^--N kg^{-1}). The CEC is relatively high (6.0 cmol kg^{-1}). Texture is dominated by clay (24.06% sand, 27.56% silt, and 48.38% clay). The soil bulk density is 0.8 g cm^{-3}. 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 as described previously (Rondon *et al.* 2007) and has been added locally around coffee plants in a long term experiment to asses biochar effect on coffee production. The soil used in our experiment was sampled during the rainy season, in July 2006, two years after the start of the field experiment. Our NB treatment consisted in soil collected form the control (biochar) treatment of the field experiment. Taken together, the soil of our B treatment contained 25.5 g of biochar by kg of dry soil.

Plant growth and experimental design

Rice plants (*Oryza sat*iva cv. Linea 30) (Chatel M 2000) were grown in greenhouses for three months under controlled conditions (temperature 27-29°C, relative humidity 65-95%, light intensity of 600 $\mu mol.m^{-2}s^{-1}$ and a 12-h photoperiod). 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 *P. corethrurus* (initial fresh weight 5 +/0.5 g), an endogeic species common in all humid tropics (Lavelle *et al.* 1987). Microcosms were regularly weeded during the experiment and maintained at 80% soil field capacity (this was checked through regular weighing of the pots). Pots were arranged in a completely randomized design. Five replicates were implemented per treatment. Plants were submitted to the four combinations between two factors: with or without biochar (B vs. NB) and with or without earthworms (E vs. NE). This resulted in four combinations of treatments: Earthworm and Biochar (EB), Biochar (B), Earthworm (E) and a Control (C) without biochar nor earthworm.

Macroscopic measurements

The macroscopic parameters considered were the following: shoot biomass, root biomass, number of leaves, number of dead leaves, total foliar area, chlorophyll concentration, leaf N concentration, leaf C/N, total N content, nitrate and ammonium. At the end of the plant cycle (110 days), plant biomass (i.e. shoot and root mass) was measured. Each plant biomass was put in a paper bag and dried in an electric oven at 40°C for 2 days.

The dry biomasses were then measured. Sub-samples of shoot biomass were analyzed for total carbon, total nitrogen and C/N (Thermo Finnigan Flash EA1112). Leaf area and Chlorophyll concentration were measured after the harvest with a leaf area meter (LI-1300 Area meter) and Chlorophyll Meter (Minolta SPAD 502) respectively. At harvest time, a final soil sample was collected for chemical analysis. Nitrate and ammonium were quantified colorimetrically using a segmented flow analyzer (Skalar Autoanalyzer, Skalar, The Netherlands).

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 50mM 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 (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 100mM citrate buffer for the acidic pH range (pHs 2.7, 4.2 and 5.2) or 50mM Tris-HCl buffer for near neutral pH (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 11000 rpm. The proteolytic activity was followed by the decrease in absorbance at λ=280 nm of the TCA soluble fraction.

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.

Semi-quantitative 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 either the cellular proteolytic activities and/or 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 2000).

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.

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		
OsSP1	**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'

Statistical analysis

Statistical analyses were performed using the SAS software version 6 (SAS 1990). 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, SS3). 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. Significant differences between means are marked by different letters in the histograms.

5.4 Results

Macroscopic effects of biochar and earthworms

Both biochar and earthworms increased soil nitrate and ammonium contents (Fig.1) but the earthworm effect was more dramatic (+92% for nitrate and +80% for ammonium) than the biochar effect (+44% for nitrate and +20% for ammonium) (Table 2). Similarly, regarding shoot biomass and the number of leaves, the effect of earthworms and biochar were both significant, but the effect of biochar was stronger than the effect of earthworms (+163% versus +98% and +87% versus +3%, for the shoot biomass and the number of leaves respectively). Although a significant interaction between biochar and earthworms was found for the number of leaves, biochar and earthworm effects were mostly additive (Table 3, Fig. 2): 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 parameter was a positive earthworm effect (+58%) (Table 3, Fig 2). There was only a significant positive biochar effect on the number of dead leaves (+100%). There was a earthworms significant effect on the total foliar area (+89%) (Table 3, Fig 2).

As shown in Figure 3, earthworms had a positive effect on leaf N concentration and on total N content (+71 and +133%), which in turn led to a negative effect on the leaf C/N (-40%) (Table 4). Similarly, biochar significantly decreased leaf N concentration (-35%) and increased total N content and leaf C/N (+76% and +45%). There were no significant interactions between earthworm and biochar for the leaf N, C/N and total N content (Table 4).

Table 2. ANOVA table of F value for the effects of biochar (B) and earthworms (E) and their interaction on nitrate and ammonium. Total df =15 *, p<0.05; **, p<0.01; ***, p<0.001; NS not significant.

	df	Nitrate	Ammonium
B	3	**19.20***	**10.88***
E	3	**75.46*****	**45.62*****
E*B	3	0.18 NS	**4.61*****
R^2		0.922	0.884

Table 3. ANOVA table of F value for the effects of biochar (B) and earthworms (E) and their interaction on shoot biomass, root biomass, number leaves, number dead leaves, total foliar area and chlorophyll. Total df = 15. *; **; *** p<0.05, p<0.01, p<0.001, NS not significant.

	df	Shoot biomass	Root biomass	Nb Leaves	Nb dead leaves	Total foliar area	Chlorophyll
B	15	**191.44*****	0.19 NS	**146.57 *****	**15.16 ****	0.04 *	0.23 NS
E	15	**78.31*****	**13.75 ****	**11.06****	1.68 NS	**11.71 ****	4.11 *
E *B	15	1.82 NS	0.29 NS	**9.36 ****	0.42 NS	0.23 NS	1.38 NS
R^2		0.94	0.47	0.91	0.51	0.42	0.26

Table 4. ANOVA table of F value for the effects of biochar (B) and earthworms (E) and their interaction on N Leaf, Leaf C/N and Total N content. Total df = 15. *; **; *** p<0.05, p<0.01, p<0.001, NS not significant.

	df	N Leaf	Leaf C/N	Total N content
B	15	**3.92 ***	**10.61 ***	**34.08*****
E	15	**176.77*****	**19.28 *****	**83.70.*****
E *B	15	0.03 NS	0.61 NS	2.93 N.S.
R^2		0.851	0.71	0.90

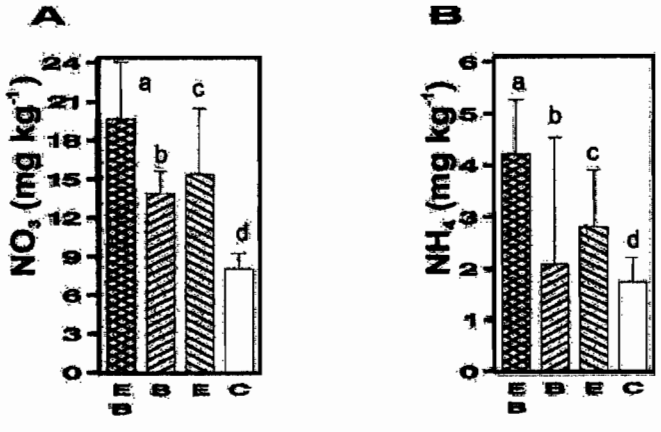


Fig.1. Effects of Biochar (B) and Earthworms (E) and Control (C) on (a) nitrate and (b) ammonium. Significant differences between means are marked by different letters (least square means).

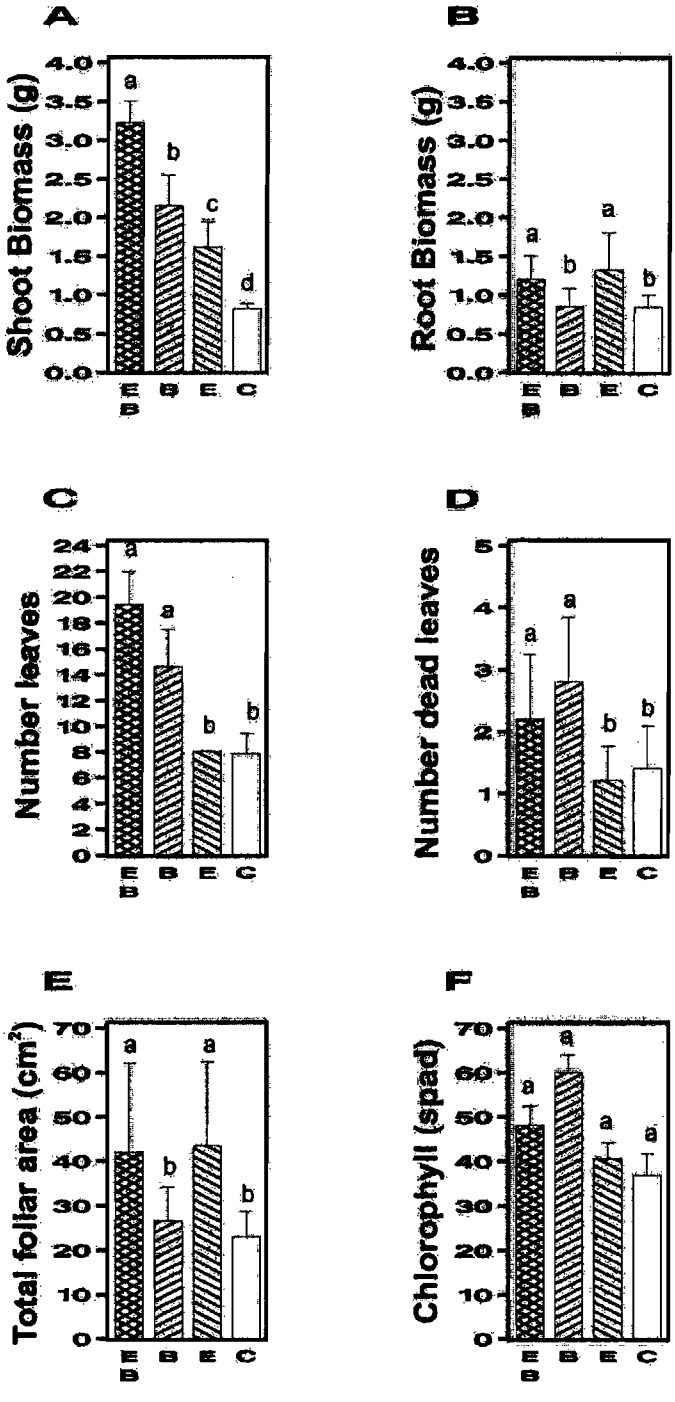


Fig.2. Effects of biochar (B) and earthworms (E) and control (C) on (a) shoot biomass, (b) root biomass, (c) number leaves, (d) number dead leaves, (e) total foliar area and (f) chlorophyll. Significant differences between means are marked by different letters (least square means).

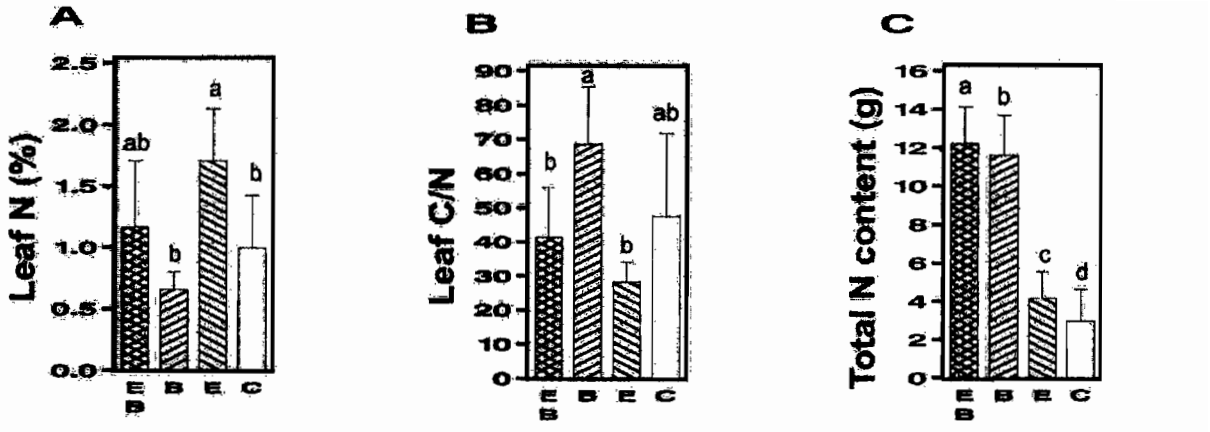


Fig.3. Effects of biochar (B) and earthworms (E) and control (C) on (a) leaf N content, (b) leaf C/N and (c) total N content. Significant differences between means are marked by different letters (least square means).

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 pH. Different pHs were used in order to have an range of different pH optima of the different classes of proteases (Rawlings 2008). The biochar treatment led to a significant raise in overall proteolytic activity, independently of the pH tested (Fig. 4). On the other hand, earthworms did not have a significant effect on leaf proteolytic activity (Fig. 4). When earthworms and biochar were used in combination, the proteolytic activities were significantly higher than the control treatment but slightly lower than when biochar was used alone (Fig. 4).

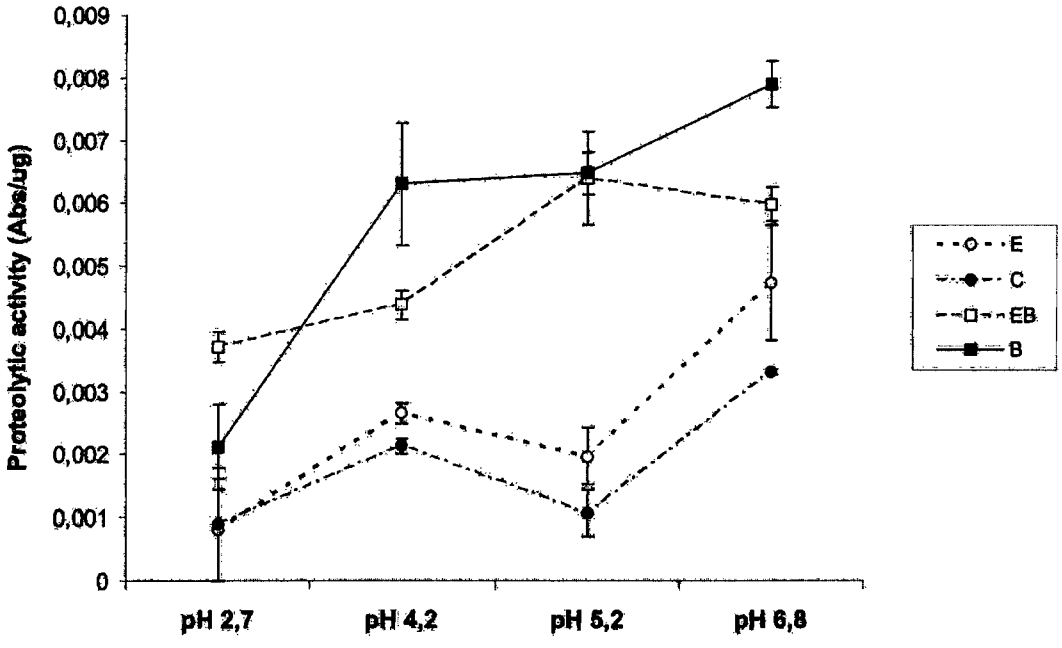


Fig. 4. Leaf proteolytic activity under different pH in of *Oryza sativa* (Linea 30) submitted to four soil treatments: Earthworm * Biochar (EB), Biochar (B), Earthworm (E) and a Control (C) without biochar and earthworm. Proteolytic activity was measured using BSA as a substrate and the assay was performed as described under Materials and Methods.

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

RT-PCR analysis showed that the biochar treatment led to a significant increase on the expression levels of three out of the six genes tested related to protein catabolism (proteases). These include one of the serine protease genes, *OsSP2* (+24%), one of the aspartic acid protease gene, *Oryzasin* (+162%) and one of the cysteine protease gene *OsCatB* (+257%) (Fig. 5 c, d, and g) (Table 5). Earthworms, on the other hand had a smaller effect on the gene expression levels of the proteases tested (Fig. 5). 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 e and g) (Table 5). 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%) and also significantly increased the expression levels of one of the cysteine protease genes, *OsCatB*

(+328%) (Fig. 5 d, e and g) and significant interactions between biochar and earthworms in Table 5).

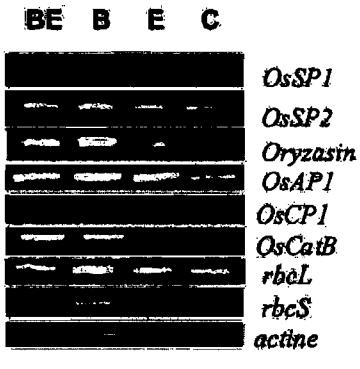
Regarding the two genes tested related to protein anabolism (rubisco subunits), *rbcS* and *rbcL*, biochar induced an up-regulation of the expression level of both (+33% and +30%, for *rbcS* and *rbcL*, respectively) (Fig. 5 h and i) (Table 5). Earthworms, on the other hand led to a decrease of the expression level of the gene coding for the small subunit of rubisco, *rbcS* (-33%) and a slight increase of the *rbcL* (+9%) gene expression level (Fig. 5 h and i) (Table 5). The significant interaction between biochar and earthworms led to a decrease of the expression levels of both *rbcS* and *rbcL* (-46% and -6,5%, respectively) (Fig. 5 h and i) (Table 5).

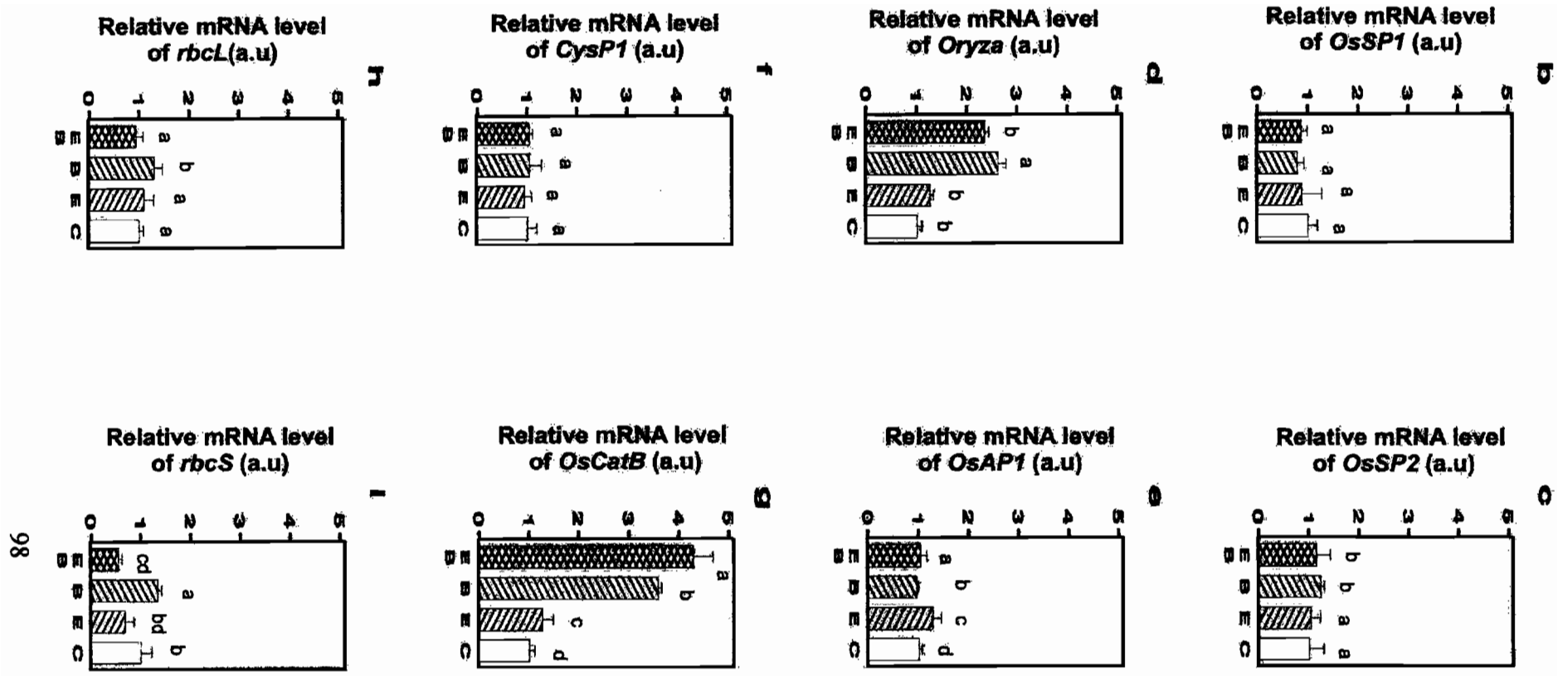
Table 5. ANOVA table of F value for the effects of biochar (B) and earthworms (E) and their interaction on Aspartic proteases: *OsSP1, OsSP2, Oryzasin, OsAP1, CysP1* and *OsCatB* and rubisco: *rbcL* and *rbcS* Total df = 3. *; **; *** p<0.05, p<0.01, p<0.001, NS not significant.

	df	*OsSP1*	*OsSP2*	*Oryzasin*	*OsAP1*	*CysP1*	*OsCatB*	*rbcL*	*rbcS*
B	3	3.89 NS	**11.96***	**2744.19*****	**220.04 *****	2.80 NS	**2726.46*****	3.92 NS	**7.56 ***
E	3	0.29 NS	0.27 NS	0.08 NS	**258.69 *****	0.59 NS	**81.94 *****	**14.33 ****	**249.67***
B*E	3	3.23 NS	1.27 NS	**104.27 *****	**52.21 *****	0.52 NS	**17.66 *****	**39.33 *****	**42.16 ***
R^2		0.480	0.62	0.997	0.985	0.328	0.997	0.878	0.973

Fig. 5. 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: EB, earthworm and biochar; B, biochar; E, earthworm; and C, control. a) Agarose gel electrophoresis; Relative mRNA level of (b) *OsSP1*, (c) *OsSP2*, (d) *Oryzasin*, (e) *OsAP1*, (f) *CysP1*, (g) *OsCatB*, (h) *rbcL*, (i) *rbcS and* (j) *actine* as the constitutive control. The mRNA levels were quantified with the Biorad QuantityOne software. Results are representative of 3 independent assays with 3 biological replicates and are means ± s.d. Significant differences between means are marked by different letters (least square means, SAS).

a)




Relative mRNA level of rbcL(a.u)
Relative mRNA level of CysP1 (a.u)
Relative mRNA level of Oryza (a.u)
Relative mRNA level of OsSP1 (a.u)
Relative mRNA level of rbcS (a.u)
Relative mRNA level of OsCatB (a.u)
Relative mRNA level of OsAP1 (a.u)
Relative mRNA level of OsSP2 (a.u)

5.5 Discussion

As far as the macroscopic variables are concerned, both earthworms and biochar had a positive effect on the growth of rice and more particularly on the shoot biomass production (Fig. 2). However, when looking at the other variables, more complicated patterns appear. For example, earthworms clearly increased root biomass but not the number of leaves whereas biochar had the opposite effect (Fig. 2). This shows that earthworms and biochar influence biomass production but also resource allocation, i.e. here, earthworms decreased the shoot root ratio and biochar had again the opposite effect. While such changes in resources allocation have already been observed, at least in the case of earthworms (Lambers *et al.* 1988c; Scheu 2003; Laossi *et al.* 2009a), they are so far difficult to interpret thoroughly because earthworms influence plants through many mechanisms (Scheu 2003; Brown *et al.* 2004a) difficult to disentangle (Blouin *et al.* 2006). Effects of biochar on resource allocation have so far been poorly studied (Lehmann *et al.* 2003b; Glaser & Woods 2004).

Here, we have shown that, indeed, both earthworms and biochar increase the availability of mineral nutrients which suggests that these mechanisms, most commonly cited both for earthworms and biochar, have played an important role in our experiment and explain partially the macroscopic effects observed in terms of biomass. However, the fact that earthworms decreased the shoot/root ratio and that biochar had the opposite effect, suggests that other mechanisms are involved which differ between earthworms and biochar. The increase in root biomass in 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 the variations in nutrient availability in space and time (Campbell *et al.* 1991; Scheu *et al.* 1999; Kreuser *et al.* 2004). In this case, it is surprising that biochar, which also increased mineral nitrogen availability (although less clearly for ammonium), did not have the same effect. 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, implemented in the same conditions as the present one, compared the effects of earthworms and biochar on rice growth in different soil types, with and without mineralization. The outcome of this experiment (Noguera, unpublished results), as well as that of a former experiment (Blouin *et al.* 2006) supports the hypothesis of plant growth factors effects.

Earthworm and biochar effects on plant nitrogen content 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. Second, while earthworms increased the leaf N concentration (therefore decreasing the leaf C/N), as has often been reported (Lambers *et al.* 1988c; Ingestad & Agren 1992), biochar had the opposite effect (Fig. 3). 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, somehow, 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 highly 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. 4) and at the molecular level by the up-regulation of the expression level of several genes related to protein catabolism and anabolism (Fig. 5). 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 tested (Fig. 2). This was also true for the number of dead leaves, which 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 of the plant (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 raise in protein degradation could be mainly related to enhanced gene expression of *Oryzasin* and *OsCatB* (Fig. 5 d and g). These genes code for an aspartic acid

protease and a cysteine protease, respectively. 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 & Faro 2004) and during leaf senescence (Hortensteiner & 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%) (Fig. 3). This could suggest that some form of N starvation could be occurring at the leaf level. Previous works have shown that under low N input, there is an increase of proteolytic events (Davies & Humphrey 1978; Cooke *et al.* 1979). Interestingly, biochar treatment also enhanced the expression of the two genes coding for the small and large subunits of rubisco, *rbcS* and *rbcL* (Fig. 5 h and i). Although in the present study we did not quantify the actual amount of rubisco synthesized in response to the different treatments, it has been shown 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 (and rubisco should be on of the main targets of leaf proteolysis since it is the most abundant leaf protein), there was simultaneously some rubisco synthesis occurring that counteracted its degradation. This would help sustain photosynthesis functioning. The increase in leaf C/N in response to biochar further supports this. This indicates that in presence of biochar there is an accelerated N metabolism as seen by the increased protein turnover that should therefore be responsible for 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 & Humphrey 1978; Cooke *et al.* 1979), it also leads to a slower growth rate and decreased biomass production (Humphrey & Davies 1975).

Earthworms, on the other hand, had much less significant effects on protein turnover. Furthermore, when earthworms were combined to biochar, they seemed to attenuate the biochar effects on protein turnover, slowing down protein degradation. 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 allows resources to be directed towards leaf production with a greater leaf area, a decreasing number of leaves and smaller shoot biomass. Nevertheless, our results suggest that the allocation of

resources due to earthworms is not directly invested in plant aboveground biomass production. 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 a 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 can 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 now needed to support this hypothesis. The enzymatic activity and gene expression levels of the enzymes involved in N uptake and metabolism like nitrate reductase, glutamine synthetase and glutamate synthase 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 then be only due to the fact that earthworms lead to the release of plant growth factors. Since biochar also influences soil microbial communities (Pietikäinen & 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 microorganism, while is likely to be the case for earthworms. It is thus well conceivable that earthworm traits allowing them to stimulate the release of plant growth factors have evolved under natural selection, maybe because earthworms would benefit of better growing plants. Although such hypotheses have never been thoroughly tested (Barot *et al.* 2007a) they are at least supported by a model (Barot *et al.* 2007b).

The contrasted effects of biochar and earthworms on N metabolism reminds of *r* and *K* – selected species as described by ecological theory (Pianka 1970; Begon *et al.* 1990) . Under high level of competition, evolution should select for organisms that have high efficiencies to capture resources when they are scarce, grow slowly, have long life cycles...etc. Under low level of competition, evolution should on the contrary select for opportunist strategies, i.e. species that are efficient at capturing resources when abundant,

growth quickly, and have short life-cycles. The first analogy between our results and the *r-K* theory is that *r* species should have high metabolic rates while *K* species should have lower metabolic rates, as found here for N metabolism in the presence of biochar and earthworms respectively. The second analogy is that *r* and *K* strategies can both be efficient depending on the ecological context, while our results show that both an increase in the protein turnover and the maintenance of a standard protein turnover may allow an efficient increase in biomass production, depending on soil conditions.

More generally, the *r/K* theory has so far been applied to whole species or populations assuming that the whole range of possible strategies is constrained by underlying trade-offs in resource allocation, and thus metabolic pathways. Here, we show that a species (or a genotype) can shift its strategy along the *r/K* gradient in a plastic way according to its environment. In physiological terms, these plastic shifts in metabolic pathways under a changing environment are called plant acclimation. Acclimation has already been extensively documented in the relevant literature (Larcher 2003) but the importation of a theory built at the species scale in another field of natural sciences might be fruitful for plant physiology. Conversely, 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 has currently been shown in microbiology (Novak *et al.* 2006). Here, in presence of biochar, plants renew faster their leaves to sustain photosynthesis, which both leads 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. Indeed both protein degradation and synthesis require respiration energy (Vierstra 1993). We could hypothesize that a higher protein turnover rate should have a higher respiration cost for rice plants grown in biochar. This could be precisely measured the future. Finally, we have shown that according to environmental conditions, plants shift their metabolism on the gradient between *r* and *K* metabolisms and, in the future, environmental features could be classified according to the position on this physiological gradient they impose to plants. Similarly, genotypes or crop cultivars could be classified as more or less prone to *r* and *K* metabolisms.

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CHAPITRE 6:

EFFETS DES VERS DE TERRE SUR LA FORMATION DE LA TERRA PRETA:

"EFFECTS OF EARTHWORM-BIOCHAR INTERACTIONS ON SOIL FERTILITY"

6 Effets des vers de terre sur la formation de la terra preta: "Effects of earthworm-biochar interactions on soil fertility"

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(Article finalisé, en attente de soumission au Plant and Soil)

6.1 Abstract

Incorporating biochar in soils and maintaining high earthworm biomasses are promising possibilities to increase the sustainability of tropical soil fertility. The effects through which earthworms and biochar influence plant growth are partially the same. Consequently, biochar and earthworm could have a synergetic effect on fertility. For example, earthworms enhance mineralization while biochar increases nutrient retention, so that the combination of the two could build up the stock of available mineral nutrients. Moreover, earthworms ingest biochar so that they could play a role in the fragmentation of biochar particles and their burrowing in the soil, which are two important processes in the maturation of biochar-enriched soils. These hypotheses were tested in a two-year mesocosm experiment using *Brachiaria humidicola* to reveal the influence of treatments on soil fertility. The grass was regularly harvested to simulate grazing. A fertilization and no-fertilization treatments where compared. Biochar had a significant effect on some soil properties but had a limited effect on *B. humidicola* growth. Its clearer effect was an increase in water retention. Earthworms increased the burrowing of biochar particles but did not markedly increase the effect of biochar on fertility. Overall, these results do not support our hypotheses. This suggests that new long-term experiments are necessary to better determine the dynamics of biochar effect on fertility. The results also show that the interaction between biochar, the regime of plant harvesting and biomass exportation should be analyzed in the future.

Key words: Biochar, earthworms, mesocosms, plant growth, *Pontoscolex corethrurus*, *terra preta nova.*

6.2 Introduction

In tropical areas where crop production is limited by low soil quality, the development of techniques improving soil fertility without negative environmental effect is a priority (Tiessen *et al.* 1994; Zech *et al.* 1997). The fertility of highly weathered soils in the humid tropics is constrained by low retention of nutrients, due to strong precipitations and the lack of stabilizing minerals, and by the rapid decomposition of organic matter (SOM) due to high temperatures and, again, precipitations (Lehmann *et al.* 2003b; Glaser 2007). New agricultural techniques based on the management of soil biota are proposed to increase the sustainability of crop yield (Lavelle *et al.* 1989; Beare *et al.* 1997). Other techniques are based on the addition of biochar and the creation of new *terra preta* soils (Glaser *et al.* 2002; Lehmann *et al.* 2003c; Lehmann & Rondon 2006; Glaser 2007).

Methods using soil fauna activity were developed to improve crop production by stabilising nutrients and organic matter within soil biogenic structures (Lavelle *et al.* 1994; Lavelle 1999; Senapati *et al.* 1999; Lavelle *et al.* 2001). In particular, earthworms have a strong impact on soil (Lavelle & Spain 2001; Wardle *et al.* 2004; Bardgett 2005) properties. Moreover, they have been shown to directly increase plant growth in 75% of the short term experiments that have compared plant growth in their presence and absence (Brown *et al.* 1999). Five mechanisms have been shown to be involved in earthworm positive effects on plant growth (Scheu 2003; Brown *et al.* 2004a): (1) increased mineralization of soil organic matter, which increases nutrient availability; (2) production of plant growth substances via the stimulation of microbial activity; (3) biocontrol of pests and parasites; (4) stimulation of symbionts and (5) modification of soil porosity and aggregation which induces changes in water and oxygen availability to plants.

Researches on tropical soils have indicated that biochar amendments can increase and sustain soil fertility (Lehmann *et al.* 2003a; Steiner *et al.* 2007; Chan & Xu 2009; Lehmann & Joseph 2009b). The beneficial effects appear to be related to an amelioration of soil physical, chemical, and biological properties: reduced acidity (Lehmann *et al.* 2003a; Topoliantz *et al.* 2005), increased cation exchange capacity (CEC) (Cheng *et al.* 2008), enhanced nitrogen (N) retention (Lehmann *et al.* 2003a; Steiner *et al.* 2008b) and increased microbiological activity (Birk *et al.* 2008; Steiner *et al.* 2008a). Biochar has also the advantage to be recalcitrant to decomposition (Seiler & Crutzen 1980), and may persist for hundreds or thousands of years, so that its effect on fertility should be long-lasting. The application of biochar is historically not a new concept and re-emerged as a promising

practice after the discovery of the Amazonian *terra preta* soils. These soils have probably been created by pre-Columbian human groups (Lehmann *et al.* 2003c; Glaser & Woods 2004). They are characterized by high contents of fragmented biochar and have remained highly fertile for hundreds of years (Lehmann *et al.* 2003c). The creation of new *terra preta* soils ('Terra Preta nova') could be the basis for sustainable agriculture in the humid tropics (Lehmann *et al.* 2003c; Glaser 2007; Lehmann 2009). For example, replacing slash and burn by slash and char could help securing food production of fast growing populations (Glaser *et al.* 2002). However, besides the addition of biochar, terra preta seem to need to maturate during at least several years (Lehmann *et al.* 2003c) to reach their high fertility and the factors enhancing this maturation are so far not well understood (Glaser & Woods 2004).

Whereas the importance of biochar for soil fertility has often been reported (Glaser *et al.* 2001; Topoliantz *et al.* 2002; Lehmann *et al.* 2003a), no published study has yet tested whether earthworms influence the formation of *terra preta.* However, biochar and earthworms have been shown to directly interact (Topoliantz *et al.* 2002; Topoliantz & Ponge 2003; Van Zwieten L. *et al.* 2009): earthworms ingest biochar, and transport biochar particles into the soil. Earthworms have thus been hypothesized to facilitate the formation of *terra preta.* (Ponge *et al.* 2006). In the present work we tested for the first time this hypothesis.

In a mesocosm experiment in semi-controlled conditions, we studied over two years the effects of earthworms on the formation of *terra preta nova* using *Brachiaria humidicola* (Rendle) Schweick as an indicator of fertility. The effects of mineral fertilization have been shown to be amplified by the addition of biochar (Steiner *et al.* 2007) and the effect of earthworm is also likely to change with the addition of mineral nutrients (Blouin *et al.* 2006; Laossi *et al.* 2009a). A full factorial experimental design was thus implemented with earthworms, biochar and mineral fertilization as factors. The general idea is that biochar increases the retention of mineral nutrients (Lehmann *et al.* 2003c) while earthworms (Lavelle 1999) and fertilization increase the availability of mineral nutrients so that the two types of processes could interact in a synergetic way to build up soil nutrient content over time (Barot *et al.* 2007b; Boudsocq *et al.* 2009).

Earthworms are also likely to influence directly nutrient retention either positively or negatively (Domínguez *et al.* 2004; Sheehan *et al.* 2006) and may thus modify on the long-term the soil nutrient content (Barot *et al.* 2007b) in interaction with mineral fertilization (Laossi 2009). Besides testing whether earthworms facilitate the formation of *terra preta nova*, we specifically addressed the following questions: (1) Biochar addition is expected to

have a positive effect on soil fertility and plant growth but what is the temporal dynamics of this effect? Does this effect start immediately? Does it increase over time during the whole two-year experiment? (2) Can earthworm, generally observed positive short-term effect on plant growth maintain over two years? (3) Does the effect of biochar addition on soil properties and plant growth increase with earthworm presence? (4) Does bioturbation by earthworms help spreading downwards in the soil profile the effect of biochar addition on soil properties? (5) Does the effect of biochar increase with mineral fertilization?

6.3 Material and Methods

Experiment set up

The experiment was conducted at the CIAT (Centro Internacional de Agricultura Tropical) in Cali, Colombia. Mesocosms were kept under a transparent roof (without walls) to allow controlling their watering and avoiding greenhouse drawbacks such as difficulties to control temperature. Three factors were implemented in a complete factorial design: presence/absence of earthworms (E / NE), addition/no addition of biochar (B / NB), addition/no addition of fertilizer (F / NF). Five replicates of each treatment combination, i.e. fertilization x soil x earthworms x biochar, were implemented. A total of 40 mesocosms were thus filled with 40 kg of dried soil.

We set up mesocosms consisting of plastic PVC pots (diameter 50 cm, height 45 cm). Drains at the bottom of pots were covered with 1 mm plastic mesh to prevent earthworms from escaping. Soil was collected at Pescador in San Pedro farm (Cauca, Colombia). The soil was collected from the top 0 -10 cm. It is a volcanic-ash soil, an inceptisol, in the USDA classification system (USDA, 1998). The soil is moderately acid: pH (H_2O) = 5.1. It is relatively rich in organic matter MO (11.5 %), and mineral nitrogen (12.9 mg NH_4^+-N kg^{-1}, 27 mg NO_3^--N kg^{-1}). The CEC is relatively high (6.0 cmol kg^{-1}). Texture is dominated by clay (24.1% sand, 27.5% silt, and 48.4% clay). The soil bulk density is 0.8 g cm^{-3}. Before the experiment, the soil was air-dried. Roots, visible plant residues and original macrofauna were removed by sieving (2 mm mesh).

The fertilized treatment (F) consisted in a unique NPK treatment at the beigning of the experiment. This treatment was adjusted to mimic fertilization practices in Colombian pastures (Thomas 1995) and corresponded to a NPK treatment of 80, 40 and 40 kg ha^{-1}

respectively. N was provided as urea. Biochar was prepared at the CIAT as described previously (Rondon *et al.* 2007). Biochar was then ground to pass through a 2 mm sieve. It was mixed with the first 5 cm of soil in biochar treatments. For the earthworm treatment, each mesocosm received ten adults (initial total fresh weight 4.0 +/0.5 g) of *Pontoscolex corethrurus* (Glossoscolecidae). Earthworms were collected at the Pescador site (Cauca, Colombia). *P. corethrurus* is an endogeic species which has a circum tropical distribution and whose geographical origin seems to be the North or north-east part of South America (Lapied & Lavelle 2003). This species can feed on soil with a low organic matter content (Lavelle *et al.* 1987).

Before starting our experiment, mesoscosms were watered to field capacity. Two days after introducing earthworms (in the treatments requiring earthworms), two plants of *Brachiaria humidicola* were sown. After one week, only one plant was kept in each mesocosmes that were regularly subsequently weeded during the experiment. They were watered with 3 l two times for week. Their position was randomized every three months to avoid confounding factors. The experiment lasted for 24 months.

Sampling

All treatments were first evaluated 12 weeks after initiating the experiment. Subsequently, every three month, shoot biomass was cut at 10 cm of the soil surface. 30% of the fresh shot biomass was added to the soil surface of the mesocosm it had been harvested in and the remaining 70% were dried at 60°C for 72 h and weighed.

At the end of the experiment (week 104), plant performance (i.e. shoot and root biomass biomasses) was measured at the final harvest. Shoots and stems were cut at the soil surface and dried separately at 60°C for 72 h. Two soil cores per mesocosm (6 cm diameter x 20 cm depth) were collected. Roots from these cores were extracted by washing on a 600 µm mesh. The fresh root systems obtained were scanned and analysed using a digital image analysing system (WinRHIZO, version 2003b, Regent Instrument, Quebec, Canada). Roots within the remaining soil of the mesocosms were then separated from the soil by washing, again on a 600 µm mesh. Besides, soil was sampled at four depths (0-10 cm, 10-20cm, 20-30 cm and 30-40 cm) for analyses. Nitrate and ammonium were quantified colorimetrically using a segmented flow analyzer (Skalar Autoanalyzer, Skalar, The Netherlands). Soil total carbon and nitrogen content were measured using a ThermoFinnigan Flash EA 1112 elemental

analyzer (ThermoFinnigan, Milan, Italy). Earthworms were collected, counted and weighed during the extraction of roots.

Statistical analysis

ANOVAs were implemented using the SAS GLM procedure (sum of squares type III, SS3)(SAS 1990) . A full model was first used to test all possible factors: Biochar (B), earthworms (E) and fertilizer (F) ("B", "E", and "F") and all, two-fold and three-fold, interactions between these factors. Since significant interactions between biochar and fertilization and earthworms and fertilization were detected, data were reanalysed separately for each fertilization treatment (Table 1, 2, 3 and 4). Presenting separate models for each treatment allows displaying the results in a more pedagogic way. To determine the direction of significant effects, we used multiple comparison tests based of least square means (hereafter LSmeans, LSmeans SAS statement), taking into account the Bonferroni correction.

6.4 Results

Biochar increases significantly the shoot biomasses in January 2007, July 2007 and October 2007 in the treatment without fertilization (NF, +231%, +129% and +159%) (Fig. 1 a, c, d and Table 1). The interaction between earthworms and biochar was only significant for the shoot biomass harvested in October 2008 (Fig. 1 h and Table 1) which led to a higher biomass in the presence of both earthworms and biochar or in the control treatment than in the presence of biochar only (Table 1, LSmeans). In the fertilization treatment (F), the pattern was slightly more complicated than without fertilization. The interaction between biochar and earthworms was significant in January 2007, January 2008 and April 2008 and led to an increase (F, +71%, +6% and +17%) in the shoot biomasses in the presence of both earthworms and biochar relatively to the earthworms, biochar or control treatments (Fig. 1 a, e, f and Table 1). These effects subsequently led to a significant biochar-earthworm interaction on the sum of the harvested shoot biomasses (LSmeans comparisons show that the earthworm-biochar treatment led to higher values than the earthworm treatment, Fig. 2 and Table 1). On the contrary, there was no significant effect on the sum of the harvested biomasses in the fertilization treatment (Table 1).

There was no significant effect, neither in the fertilization nor in the no-fertilization treatment (F and NF), on global root descriptors such as the root biomasses or the shoot/root

ratios. Similarly, there was no significant effect on descriptors obtained using winRHIZO (total root length, mean root diameter, specific root length, root ramification; detailed statistical results not shown).

Table 1. ANOVA table of F values for the effects of biochar (B) and earthworms (E) and their interaction on the shoot biomass at different harvest dates (every three months) and the total harvested biomasses. The last line gives the significant LS mean differences between treatment combinations. One model has been analysed for each fertilization treatment (NF no fertilization; F, fertilization). Total df = 39. *; **; *** $p<0.05$, $p<0.01$, $p<0.001$; - , does not apply, NS not significant.

		NF								
	df	**January 2007**	**April 2007**	**July 2007**	**October 2007**	**January 2008**	**April 2008**	**July 2008**	**October 2008**	**Total sum of harvested shoot biomasses**
B	1	**5.51 ***	1.31 NS	**4.41 ***	**4.66 ***	3.07 NS	0.09 NS	0.34 NS	0.11 NS	2.00 NS
E	1	0.38 NS	0.60 NS	0.04 NS	0.44 NS	0.10 NS	2.88 NS	0.00 NS	0.10 NS	0.01 NS
E*B	1	0.39 NS	0.34 NS	0.07 NS	0.09 NS	0.00 NS	0.01 NS	0.27 NS	**8.49 ***	0.09 NS
R^2		0.28	0.12	0.224	0.24	0.16	0.15	0.03	0.35	0.11
LS means		**B>C** -	-	**B>C**	**B>C**	-	-	-	**EB,C>B**	-

		F								
	df	**January 2007**	**April 2007**	**July 2007**	**October 2007**	**January 2008**	**April 2008**	**July 2008**	**October 2008**	**Total sum of harvested shoot biomasses**
B	1	**16.36*** **	0.94 NS	0.06 NS	0.00 NS	0.03 NS	2.27 NS	0.30 NS	0.00 NS	1.70 NS
E	1	0.62 NS	0.40 NS	0.47 NS	0.33 NS	0.24 NS	0.15 NS	1.67 NS	1.73 NS	0.00 NS
E*B	1	**6.89***	1.66 NS	0.40 NS	0.02 NS	**5.09 ***	**9.60 ** **	0.92 NS	1.28 NS	**4.61 ***
R^2		0.59	0.15	0.05	0.02	0.25	0.42	0.15	0.15	0.2
LS means		**EB>E,C**	-	-	-	**EB>B**	**EB>E,C**	-	-	**EB>E**

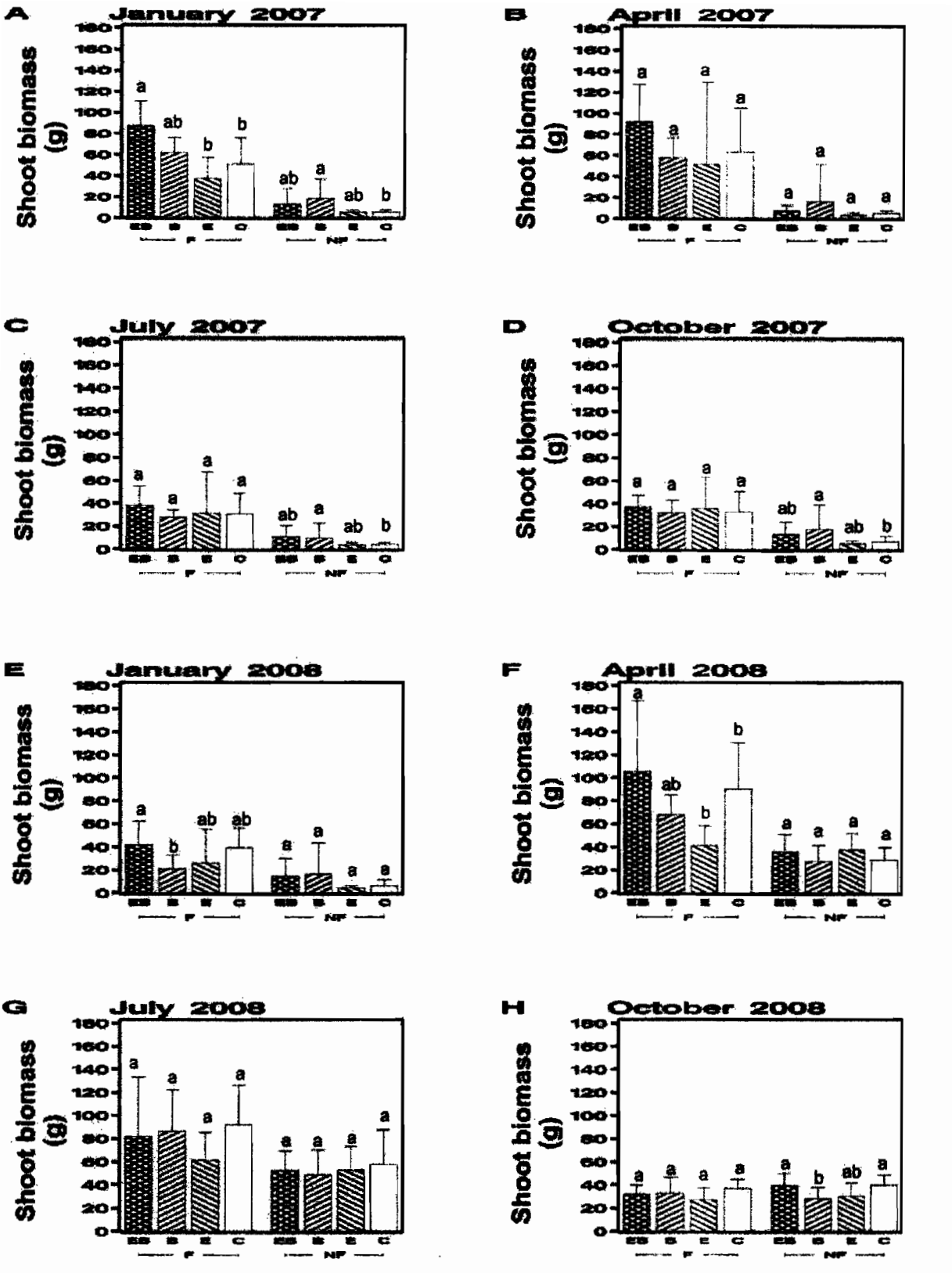

Fig.1. Effects of earthworm and biochar on shoot biomass at different harvest dates (january 2007, april 2007, july 2007, october 007, january 2008, april 2008, july 2008, october 2008). Mean values are displayed together with standard deviations. NF, no fertilizer soil; –F, fertilizer soil. Significant differences between means are marked by different letters (least square means).

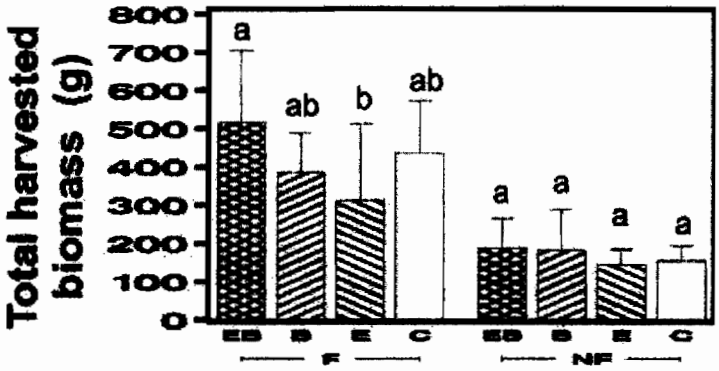

Fig.2. Effects of earthworm and biochar on total harvested biomasses. Mean values are displayed together with standard deviations. NF, no fertilizer soil; –F, fertilizer soil. Significant differences between means are marked by different letters (least square means).

Biochar reduced the volume of drained water (-289 % and - 239% respectively) in the NF and F treatments (Fig 3 c and Table 2). Biochar increased the height of soil in the mesocosms by 7%, relatively to the other treatments, only in the no-fertilization (NF) treatment (Fig 3 b and Table 2). The effects of the different treatments on the nitrate content of the drained water were quite complex (Fig. 3), i.e. the interaction between the earthworms and biochar was significant in both the fertilization and no-fertilization treatment (F and NF) (Table 2). In the no-fertilization treatment there was more nitrate in the biochar treatment than in the control treatment and the combination of biochar and earthworms (LSmeans differences, Table 2). In the fertilization treatment there was more nitrate in the treatment with both earthworms and biochar than in the three other treatments (B, E, C; LSmeans differences, Table 2).

At the end of the experiment there were approximately four earthworms in each mesocosm treatment (requiring earthworms) however in the fertilization treatment without biochar (E) the number of earthworms was significantly increased (more than twofold).

Table 2. ANOVA table of F value for the effects of biochar (B), earthworms (E) and soils (S) and their interaction on earthworms number, the soil height, the volume of drained water and the nitrate concentration in the drained water. One model has been analysed for each fertilization treatment (NF no fertilization; F, fertilization;). Total df = 23. *; **; *** p<0.05, p<0.01, p<0.001; - , does not apply, NS not significant.

		NF				F			
	df	**Earthworms number**	**Height of soil in the mesocosms**	**Volume of drained water**	**Nitrate concentration in the drained water**	**Earthworms number**	**Height of soil in the mesocosms**	**Volume of drained water**	**Nitrate concentration in t drained water**
B	1	0.23 NS	**16.65 *****	**65.80 *****	2.59 NS	**9.99 *-**	2.43 NS	**85.09 *****	**7.56 ***
E	1	-	**19.03 ****	0.06 NS	**7.19 ***	-	7.07 NS	0.50 NS	**10.79 ***
E*B	1	-	**8.73****	0.17 NS	**18.24 ****	-	1.89 NS	1.40 NS	**21.13 ****
R^2		0.17	0.73	0.80	0.77	0.55	0.41	0.84	0.83
LS means		-	**B>EB,E,C**	**C>B**	**B>EB,C**	-	-	**E,C > EB, B**	**EB>B,C,E**

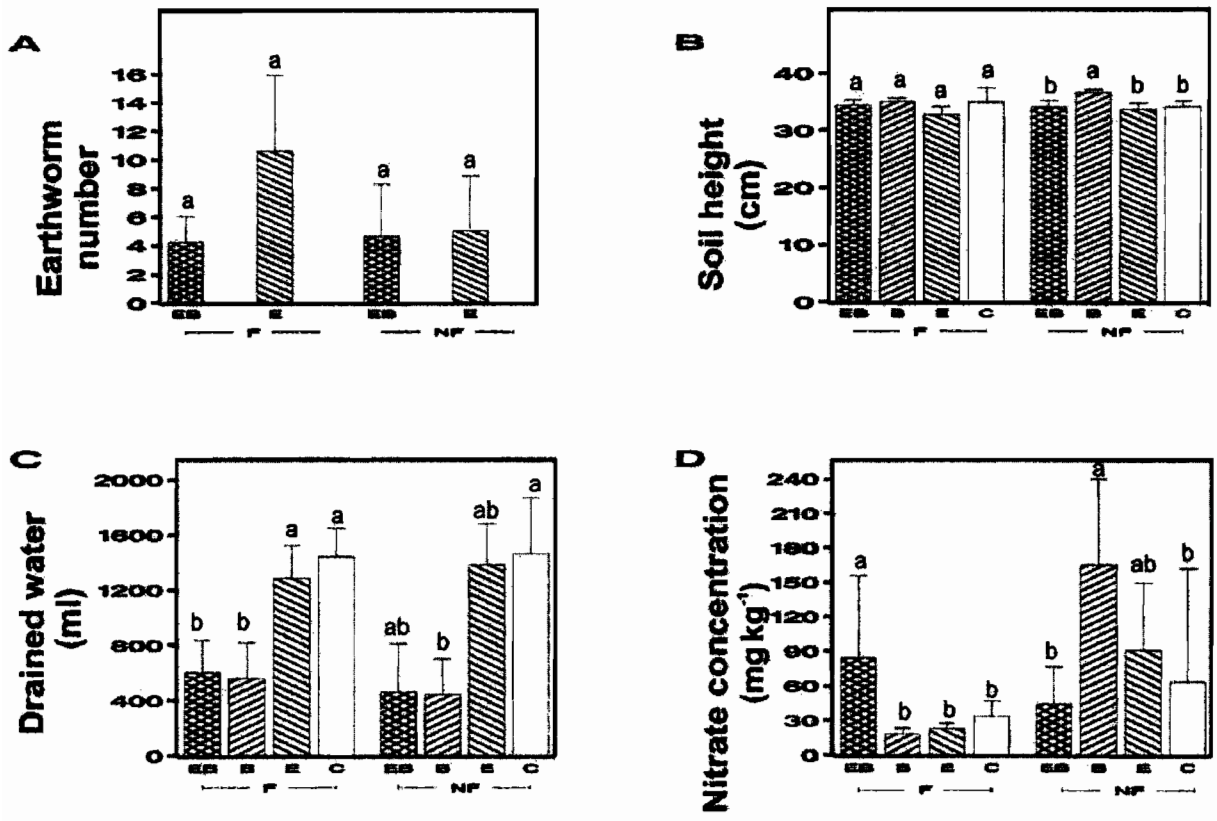

Fig.3. Effects of earthworm and biochar on (a) earthworms number (b) soil height: height of soil in the mesocosms at the end of the experiment (c) drained water: volume of drained water after 3 l of water had been purred in each mesocosm at the end of the experiment and (d) nitrate concentration: concentration in nitrate of the drained water. Mean values are displayed together with standard deviations. NF, no fertilizer soil; –F, fertilizer soil. Significant differences between means are marked by different letters (least square means).

There were few significant effects of the different treatments on the soil nitrate and ammonium contents. At 10-20 cm depth, in the no-fertilization treatment, the nitrate content was higher in the biochar treatment than in the control (+230%, Fig. 4 and Table 3, LSmeans comparison). Taken together the significant effect of biochar and earthworms led to a higher nitrate content in the biochar treatment than in the treatments with only earthworms and with both earthworms and biochar (Table 3 and Fig.4). In the fertilization treatment (F), biochar increased soil nitrate content at 0-5 cm depth (+56%, Fig 4 a and Table 3). At 20-30 cm, the significant earthworm effect and interaction between biochar and earthworms led to a higher nitrate content in the two earthworm treatments (E and EB) than in the control treatments (Fig. 4 and Table 3). The only significant effects on soil ammonium content were in the no-fertilization treatments and were consistent at the three soil depths: biochar decreased significantly the ammonium content at 0-5, 10-20 and 20-30 cm (Fig.4, Table 3, - 6%, - 46 % and - 30% respectively for the three soil depths).

Table 3. ANOVA table of F value for the effects of biochar (B), earthworms (E) and their interaction soil nitrate content and soil ammonium content. The last line gives the significant LS mean differences between treatment combinations. One model has been analysed for each fertilization treatment (NF no fertilization; F, fertilization). Total df = 23. *; **; *** p<0.05, p<0.01, p<0.001; - , does not apply, NS not significant.

		NF				F			
	df	**Nitrate 0-5 cm**	**Nitrate 5-10 cm**	**Nitrate 10-20 cm**	**Nitrate 20-30 cm**	**Nitrate 0-5 cm**	**Nitrate 5-10 cm**	**Nitrate 10-20 cm**	**Nitrate 20-30 cm**
B	1	0.30 NS	0.47 NS	2.54 NS	**4.04 ***	**11.18 ***	0.14 NS	0.58 NS	0.85 NS
E	1	1.15 NS	0.04 NS	0.01 NS	**14.59 ****	0.14 NS	1.79 NS	0.14 NS	**5.48 ***
E*B	1	1.79 NS	13.22 NS	**18.18****	0.04 NS	2.32 NS	2.70 NS	0.59 NS	**3.52***
R^2		0.28	0.63	0.72	0.70	0.63	0.36	0.14	0.55
LS means		-	-	**B> C**	**B>EB, E**	-	-	-	**EB,E>C**

		NF				F			
	df	**Amonium 0-5 cm**	**Amonium 5-10 cm**	**Amonium 10-20 cm**	**Amonium 20-30 cm**	**Amonium 0-5 cm**	**Amonium 5-10 cm**	**Amonium 10-20 cm**	**Amonium 20-30 cm**
B	1	**4.50 ***	3.27 NS	**11.48 ****	**8.09 ***	4.48 NS	1.32 NS	0.1 NS	1.40 NS
E	1	0.35 NS	0.03 NS	0.11 NS	0.82 NS	0.02 NS	1.15 NS	0.14 NS	0.26 NS
E*B	1	2.60 NS	0.09 NS	1.92 NS	0.18 NS	0.14 NS	0.50 NS	0.03NS	0.26 NS
R^2		0.48	0.29	0.62	0.53	0.36	0.19	0.46	0.19
LS means		-	-	-	-	-	-	-	-

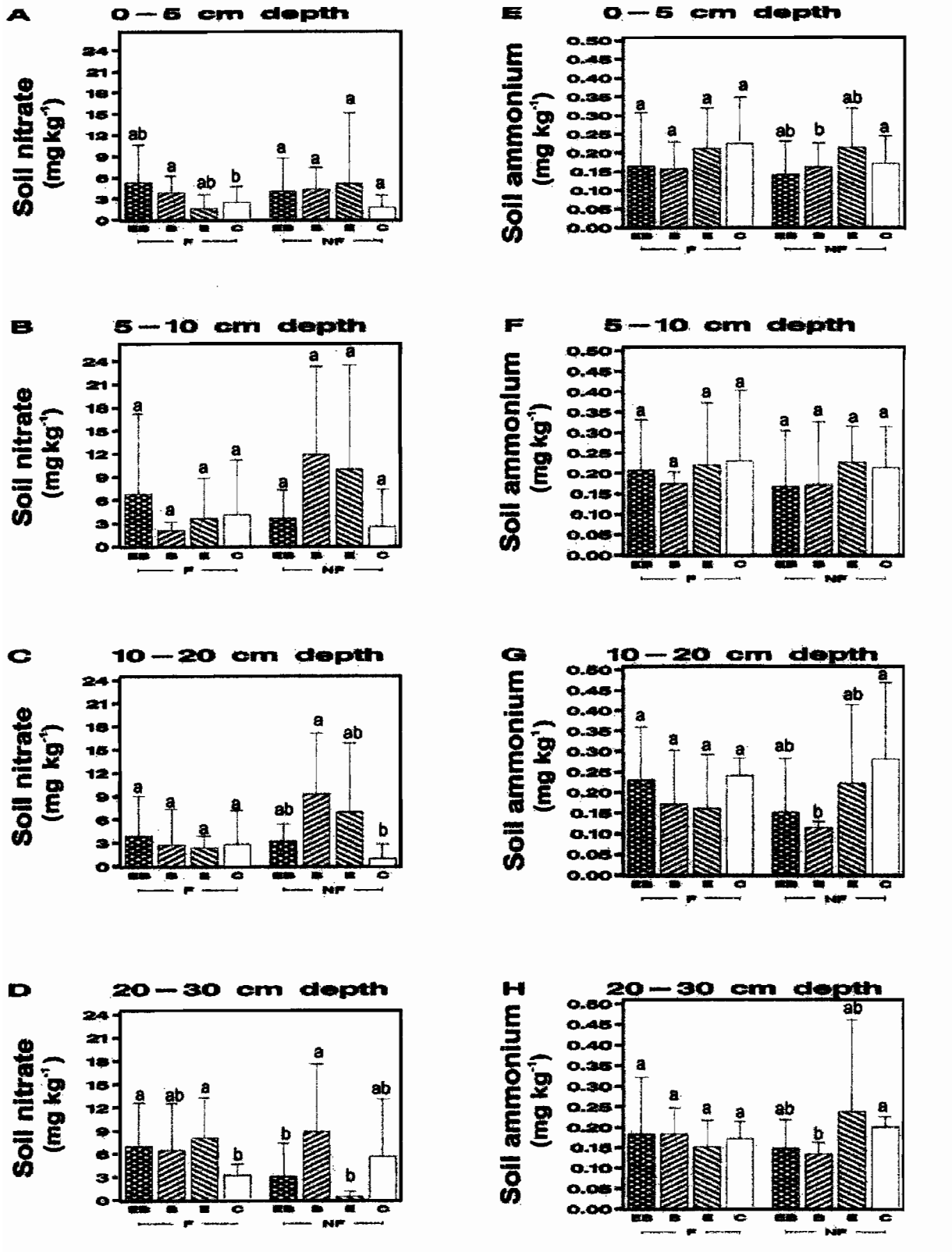

Fig.4. Effects of earthworm and biochar on (a) soil nitrate content and (b) soil ammonium content at four soil depths. Mean values are displayed together with standard deviations. NF, no fertilizer soil; –F, fertilizer soil. Significant differences between means are marked by different letters (least square means).

In the no-fertilization treatment, the soil carbon content presented a complex pattern. At both 0-5 and 5-10 cm depths, there was a dominant biochar effect (there was naturally more carbon in the presence of biochar) but in addition. Hence, there was a significant biochar-earthworm interaction resulting in more carbon in the biochar treatment than in the biochar-earthworm treatment at 0-5 cm depth and the opposite at 5-10 cm depth (Fig. 5 and Table 4) At lower depths (10-20 cm and 20-30 cm no significant effects were detected (Fig 5 c, d and Table 4). In the fertilization treatment, biochar also increased soil carbon content at the soil surface (respectively +131% and +108% at 0-5 cm and 5-10 cm, Fig. 5 a, b and Table 4) but not at lower depths and there was no interaction between earthworm and biochar treatments.

Soil organic nitrogen content followed a different pattern from soil carbon content. In the no-fertilization treatment, biochar increased (+22%) the soil nitrogen content at 0-5 cm depth (Figure 5 e and Table 4). At the three other depths the pattern was more complicated and there was a significant biochar x earthworms interaction. The general trend was that the combination of biochar and earthworms increased the nitrogen content relatively to the three other treatments (Table 4, LSmeans comparisons). In the fertilization treatment, biochar increased soil nitrogen content at 0-5 cm (+ 23%, Fig. 5 and Table 4). There was a significant interaction between earthworms and biochar at 10-20 cm depth (Table 4) and the nitrogen content was higher in the biochar treatment than in the biochar-earthworm and control treatments. There was no significant effect on soil nitrogen content at 5-10 cm and 20- 30 cm depth in the F treatment (Table 4).

Table 4. ANOVA table of F value for the effects of biochar (B), earthworms (E) and their interaction on C soil and N soil. The last line gives the significant LS mean differences between treatment combinations. One model has been analysed for each fertilization treatment (NF no fertilization; F, fertilization). Total df = 23. *; **; *** p<0.05, p<0.01, p<0.001, - , does not apply, NS not significant.

		NF				F			
	df	C 0-5 cm	C 5-10 cm	C 10-20 cm	C 20-30 cm	C 0-5 cm	C 5-10 cm	C 10-20 cm	C 20-30 cm
B	1	**139.63 ****	**78.06 *****	5.84 NS	1.80 NS	**16.16 ****	**27.58 *****	0.57 NS	2.57 NS
E	1	**12.46****	**14.35 ****	0.64 NS	0.30 NS	0.06 NS	1.84 NS	2.46 NS	0.39 NS
E*B	1	**9.69 ***	**14.76 ****	1.39 NS	22.84 NS	0.12 NS	2.05 NS	6.34 NS	2.85 NS
R^2		0.95	0.93	0.49	0.75	0.67	0.79	0.53	0.42
LS means		**EB>E,C B>EB,C**	**EB>B> E,C**	-	-	**B>C**	**B>C**	-	-

		NF				F			
	df	N 0-5 cm	N 5-10 cm	N 10-20 cm	N 20-30 cm	N 0-5 cm	N 5-10 cm	N 10-20 cm	N 20-30 cm
B	1	**20.31 ****	**68.92*****	**9.77 ***	0.31 NS	**10.03 ***	5.16 NS	0.92 NS	0.01 NS
E	1	1.68 NS	**24.22 ****	0.41 NS	0.09 NS	0.61 NS	0.45 NS	0.38 NS	0.01 NS
E*B	1	0.20 NS	**40.04 ****	**12.12 ****	**19.73 ****	0.14 NS	0.56 NS	**15.54 ****	1.58 NS
R^2		0.73	0.94	0.73	0.71	0.57	0.43	0.67	0.16
LS means		**B>C**	**EB>B,E,C B>E**	**EB>B,E,C**	**EB>B,E**	**B>C**	-	**B>EB,C**	-

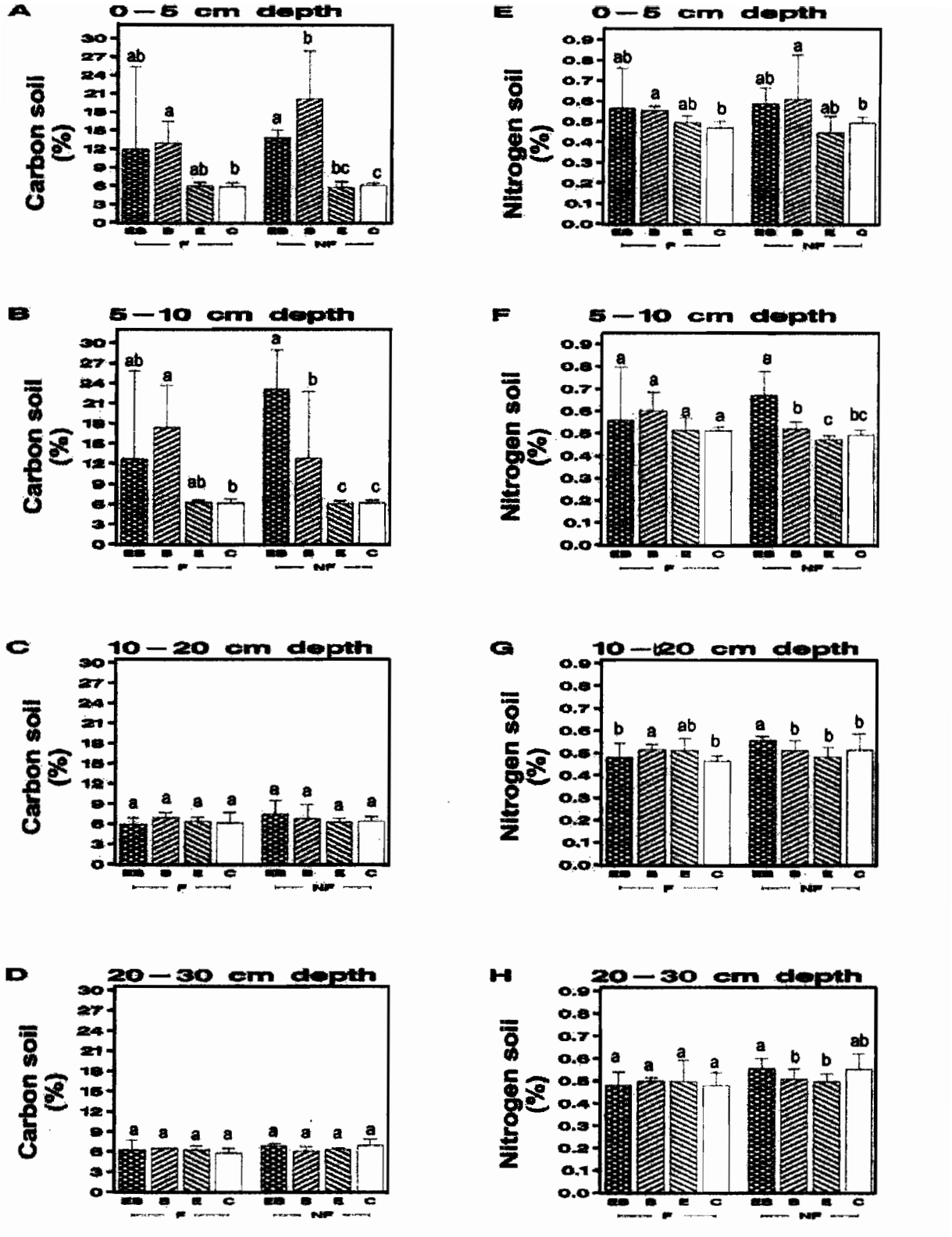

Fig.5. Effects of earthworm and biochar on (a) soil C content and (b) soil N content at four soil depth. Mean values are displayed together with standard deviations. NF, no fertilizer soil; –F, fertilizer soil. Significant differences between means are marked by different letters (least square means).

6.5 Discussion

Our present results allow answering the five questions raised in the introduction. We did find a positive effect of biochar on plant growth. However this effect was relatively small and was not consistent between all harvest dates (first question). In fact, the biochar effect on shoot biomass was significant as soon as a few months after the beginning of the experiment and tended to fade at the end of the experiment. Hence, contrary to what we expected, the effect of biochar did increase in time along a kind of maturation phase. We did not find any simple effect of the earthworms on the shoot biomass (second question), but there was however a positive effect of the earthworms on shoot biomasses in the presence of biochar in the fertilization treatment and this effect was detected as late as eighteen months after the beginning of the experiment. There were also very few effects of the earthworms on the soil properties at the end of the experiment and therefore not suggesting any improvement in the soil fertility by the earthworms. We found that, in some cases, there was a synergy between earthworms and biochar that led to an increase on the shoot biomasses. However, this effect was clearer in the fertilization treatment and only at three harvest dates out of eight (third question). There were also very few earthworm effect on the soil properties. There was mostly an increase in soil nitrate content at 20-30 cm depth in the fertilization treatment. There was also an increase in the soil carbon content at 5-10 cm depth and a decrease at 0-5 cm, but only in the no-fertilization treatment. This shows that earthworms have had a limited role in spreading biochar downwards in the soil (fourth addressed question). Finally, there was a synergetic effect of biochar and mineral fertilization in the sense that the biochar-earthworms interaction had a significant effect on shoot biomass only in the fertilization treatment (fifth question). This suggests that there is a higher level of interaction between biochar-earthworms and mineral fertilization. We discuss below these answers to the five addressed questions.

It is surprising that the effect of biochar on the plant growth and the soil properties were not very clear in our experiment. Indeed, clearer effects where found on the growth of rice in a microcosm experiment (Noguera unpublished result) and on the growth of coffee in a field experiment (Rondon, unpublished results). This might first show, as for the effect of earthworms (see below), that the effect of biochar highly depends on the combination of soil type and plant species. Studies of biochar effect on plant growth are probably in their infancy in comparison with the vast literature on plant-earthworm interactions (Alphei *et al.* 1996; Brown *et al.* 1999). It would thus be too early to predict the effect of biochar in previously

Brown *et al.* 1999). It would thus be too early to predict the effect of biochar in previously unstudied cases. Some effects of biochar on soil properties were however found. There was a negative effect of biochar on the soil ammonium content but only in the fertilization treatment, while there was a trend for an increase in nitrate content with biochar in both the fertilization and no-fertilization treatments. Biochar is supposed to increase soil capacity to retain both nitrate and ammonium (Glaser *et al.* 2002; Lehmann *et al.* 2003a; Rondon *et al.* 2007; Steiner *et al.* 2008b). An explanation of the decreased ammonium content could be that biochar does increase grass shoot production in the no-fertilization treatment, which is likely to have led to a higher ammonium uptake by *B. humidicola*, which is likely to have a high affinity for ammonium (Subbarao *et al.* 2007) This positive biochar effect on biomass production would in turn be due to its clear effect on water retention (diminution of drained water), which is in fact the clearer and more consistent effect in the whole experiment (-50%). Besides, such positive effect of biochar on water retention have already been observed (Piccolo & Mbagwu 1990; Lehmann *et al.* 2003a; Glaser & Woods 2004; Major *et al.* 2009). The fact that we did not find any effect of biochar on root and leaf C/N ratios (results not detailed) supports this rationale and the hypothesis that biochar does not increase significantly nutrient availability in our experiment.

During the two-year experiment, we did not observe any progressive increase in soil fertility contrary to the idea of *terra preta* maturation: the high fertility of *terra preta* anthroposols and the longevity of this fertility could be in fact due to many years of agricultural practices which led to a progressive increase in soil organic matter and mineral nutrients contents (Marris 2006; Glaser 2007). It is indeed possible that higher biomass productions and lower rates of mineral nutrients have a synergetic and progressive effect on soil fertility. Longer-term experiments would be required to test for this hypothesis and, to our knowledge, no such work has yet been published. Another remark is that the build up of soil fertility with the addition of biochar should also depend on the regime of organic matter (and thus nutrient) importation (Lehmann *et al.* 2003b; Glaser *et al.* 2004a; Steiner *et al.* 2007; Steiner *et al.* 2008b), as already shown, but also on exportations. Here 2/3 of standing biomass was exported every three month to simulate grazing. This exportation rate might be high relatively to what happens in real pastures, and does not take into account returns of carbon and nutrients through feces and urine. This might have prevented the building up of soil fertility. New experiments encompassing several cycles of plant growth/harvest should be preformed in order achieved to test for the interaction between biomass exportation and

biochar addition. Indeed, to increase crop production in a sustainable way high rates of carbon and nutrient exportation have to be dealt with.

Earthworm effects were less clear than expected, especially because the same earthworm species increased strongly rice (*Oryza sativa*) growth in the same soil in a microcosm experiment (Noguera unpublished results). A first explanation, as for biochar, is that there is so far no general theory allowing to predict how the effect of a given earthworm species on plant growth varies in an interactive way, with both plant species and soil properties (Doube *et al.* 1997; Brown *et al.* 1999; Laossi *et al.* 2009a). It is generally admitted that earthworms tend to have a more positive effect on grasses than on other plant functional type, because they rely on the mineralization of soil organic matter, which is clearly enhanced by earthworms (Kreuser *et al.* 2004; Wurst *et al.* 2005; Partsch *et al.* 2006). However, up to our knowledge, no study has been published on the effect of an earthworm species on the tropical perennial grass *Brachiara humidicola* (used in our experiment) and this species might have a particular response to earthworms. Moreover, it has been shown that the plant response to earthworms does not necessarily increase with grasses (Laossi *et al.* 2009a). A second explanation, for the absence of a simple earthworm effect on *Brachiara* growth is that most earthworm effects on plant growth have been documented in short-term microcosm experiments (Patron *et al.* 1999) while our experiment lasted 2 years and was achieved in larger container. Although, living earthworms were collected at the end of the experiment as well as some cocoons (data not shown), which showing that earthworms were active all through in our earthworm treatments, the earthworm densities were lower than those usually applied in microcosm experiments. In fact, while it is possible to maintain high earthworm densities (often slightly higher than densities normally recorded in the field) in microcosms during three months (often slightly higher than the densities normally recorded in the field). However, in our experiment, *Pontoscolex* densities had the time to reach, in our experiment, an equilibrium according to the soil type, the inputs of organic matter and the climatic conditions. Our result could thus be due to the fact that our experiment lasted longer (Brown *et al.* 2000; Scheu 2003) and involved more realistic earthworm densities than the usual plant-earthworm experiment. Another mesocosm experiment supports this rationale (Laossi 2009).

Our experiment showed that the biochar effect on plant growth increased in some cases with the presence of earthworms, but this effect was moderate and only occurred with mineral fertilization. They were also limited interactive effect between earthworms and

biochar on the soil properties. The clearer effect, the burrowing of biochar from the 0-5 cm to the 5-10 cm soil layers in the no-fertilization treatment, does not lead to a synergetic effect on the shoot biomass. This observation must be due to the ingestion of biochar particles by *Pontoscolex* and bioturbation (Topoliantz & Ponge 2005; Ponge *et al.* 2006; Subbarao *et al.* 2007). This shows, as predicted, that indeed biochar and earthworms interact but also suggests that earthworms are not critical for the formation of the *terra preta* contrary to some recent predictions (Ponge *et al.* 2006). In particular, there was no synergetic effect between earthworms and biochar on the availability of mineral nitrogen: the likely enhanced mineralization of organic matter by *Pontoscolex (Pashanasi et al. 1992; Brown et al. 2000; Araujo et al. 2004; Lafont et al. 2007)* does not increase the biochar effect on nitrate and ammonium retention (Lehmann *et al.* 2003a; Steiner *et al.* 2008b; Chan & Xu 2009). However, it cannot however be more once excluded that such a synergy would need more than two years to establish.

The absence of synergetic effect between fertilization and biochar shows again that increasing the nutrients inputs (as with earthworm-enhanced mineralization) and decreasing the nutrient leaching does not automatically lead to a synergetic increase in nutrient availability and primary production, contrary to the prediction of some simple models (Barot *et al.* 2007b; Boudsocq *et al.* 2009). However, we have found several significant earthworm-biochar interactions that depend on the fertilization treatment: (1) positive effect of the combination of earthworm and biochar on shoot biomass only in the fertilization treatment, (2) trend to a decrease in soil nitrate content with both earthworms and biochar only without fertilization, (3) increase in nitrate leaching in presence of both earthworms and biochar with fertilization, reverse pattern without fertilization, (4) increase in earthworm density without biochar and only in the fertilization treatment. The existence of this type of triple interaction is so far difficult to interpret. One possible hypothesis is that both earthworms and biochar influence microbial communities and that this modulates in a complex and, so far, unpredictable way plant-microorganism competition for nutrients (Hodge *et al.* 2000) and nutrient exportation through leaching and denitrification (Brown 1995; Perakis *et al.* 2005). Earthworms are indeed known to stimulate the activity of a subset of soil bacteria in their casts and may have positive (Li *et al.* 2002) and negative (Zhang *et al.* 2000) effects on soil microbial biomasses. Similarly, biochar is known, to increase microbial biomasses and is likely to change the structure of microbial communities (Pietikäinen & Fritze 2000; Steiner *et al.* 2004; Warnock *et al.* 2007; Steiner *et al.* 2008a; Thies & Rillig 2009). Interactive effects

of biochar and earthworms on the structure and activities of microbial communities, not documented here, will thus have to be documented in future experiments.

Finally, the increase in earthworm density without biochar and with mineral fertilization also remains difficult to explain. Since earthworms cannot benefit directly form mineral nutrients, this effect should be, again, due to interactions with microorganisms involving or not plant-soil feedback. For example, combination of biochar-fertilization treatments could lead to different root turnovers and inputs of organic matter (exudates) which could in turn have positive or negative effects on earthworms. More generally, our mesocosm study shows the necessity of studying in the field the long term effects of biochar and terra preta on soil macrofauna, which has so far not been achieved.

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CHAPITRE 7:

SYNTHESE ET DISCUSSION GENERALE

7 Synthèse et discussion générale

7.1 Synthèse des résultats

Les résultats obtenus au cours de cette étude permettent d'apporter des informations sur les mécanismes par lesquels les vers de terre et biochar affectent la croissance des plantes, ainsi que l'influence des vers de terre et du biochar sur la fertilité du sol dans des expériences à court et long terme. De même, cette étude permet d'apporter des éléments sur les possibles interactions qui existent entre vers de terre et biochar.

Nous avons montré à travers les différents chapitres de cette thèse que le biochar et les vers de terre affectent indépendamment la croissance des plantes, généralement positivement, mais que les vers et le biochar n'interagissent que très peu dans leurs effets respectifs. Pratiquement aucune interaction de ce type n'a été trouvée dans les expériences à court terme. Quelques interactions significatives ont cependant été trouvées dans l'expérience en mésocosmes (2 ans). Ces résultats invalident globalement l'hypothèse d'une forte synergie entre vers et biochar qui augmenterait la fertilité des sols. Ainsi, nous avons montré que l'addition de biochar aux sols et l'augmentation de la biomasse de vers de terre sont deux possibles manières d'augmenter la fertilité de sols tropicaux et de maintenir la production de cultures. Les deux méthodes peuvent être combinées dans la mesure où leurs effets sont additifs mais ma thèse suggère qu'il ne faut pas s'attendre à un renforcement mutuel de ces effets.

Un autre résultat apparait comme général dans toute ma thèse: les mécanismes par lesquels les vers et le biochar influencent la croissance des plantes ne sont pas exactement les mêmes. Le mécanisme le plus facile à mettre en évidence passe par l'augmentation de la disponibilité des nutriments minéraux, les vers de terre augmentant la minéralisation, et le biochar augmentant la rétention des nutriments. Cependant, différents effets observés sont mal corrélés aux effets des vers et du biochar sur les teneurs en nitrate et ammonium. Par exemple, les vers mais pas le biochar augmentent la biomasse racinaire et le nombre de ramifications du système racinaire quelque soit la richesse du sol en nutriments (Chapitre 4.1). Ces résultats suggèrent que d'autres mécanismes interviennent en présence des vers de terre. Comme ces mécanismes changent l'allocation des ressources du riz, il est probable qu'ils font intervenir la

production de molécules reconnues comme des phytohormones par le riz. Ces molécules pourraient être produites par des bactéries spécifiquement stimulées par les vers.

Quatre autres conclusions majeures ressortent des différentes expériences de ma thèse.

Chapitre 4.1 Les effets des vers de terre et du biochar sur la croissance du riz dépendent hautement du type de sol et de la fertilisation. Si la fertilisation minérale a tendance à diminuer la réponse du riz au biochar et au vers, la réponse ne sera pas forcément dans un sol plus riche agronomiquement (plus de matière organique, plus de nutriments minéraux) que dans un sol plus pauvre.

Chapitre 4.2 Les effets des vers de terre et du biochar sur la croissance du riz dépendent fortement de la variété de riz considérée. Les premiers résultats obtenus suggèrent que la variété la plus rustique et la moins intensément sélectionnée, *Donde lo tiren*, est celle qui répond le plus au biochar et aux vers. Cependant, le pattern des réponses est très complexe. Par exemple, une variété répondant très positivement au biochar ne répond pas forcément très positivement aux vers (et vice versa) ou à la combinaison vers-biochar.

Chapitre 5 Le biochar et les vers de terre influencent différemment le métabolisme de l'azote du riz. Dans le sol riche, où à la fois les vers et le biochar augmentent la croissance du riz, le biochar augmente à la fois le catabolisme et l'anabolisme des protéines tandis que les vers de terre ont moins d'effets significatifs sur le métabolisme des protéines. En résumé, le biochar semble induire chez le riz un métabolisme de "type *r*" alors que les vers induisent un métabolisme de "type *K*".

Chapitre 6 Dans l'expérience de 2 ans en mésocosmes, quelques interactions entre biochar et vers de terre ont été mises en évidence. Par exemple, les vers ingèrent bien le biochar et contribuent à descendre les particules de biochar dans le profile de sol. Ces dernières interactions ne paraissent cependant pas critiques pour la formation de la terra preta.

7.2 Discussion

Il peut paraitre étonnant que je n'aie pas trouvé de nombreuses interactions entre les vers et le biochar. En effet, ils agissent en partie par les mêmes mécanismes (Glaser *et al.* 2002; Brown *et al.* 2004a) et l'augmentation de la minéralisation et de la rétention des nutriments pourraient avoir un effet synergique (Barot *et al.* 2007b; Boudsocq *et al.* 2009).

De plus, comme le confirme l'expérience en mésocosmes, les vers de terre ingèrent bien les particules de biochar. Une première remarque est qu'il est difficile de généraliser les résultats de ma thèse. On sait par exemple que la réponse de différentes espèces de plantes à une espèce de vers de terre dépend énormément de l'espèce de plante, de l'espèce de vers et du sol utilisé (Wurst *et al.* 2005; Laossi *et al.* 2009a). Aucune théorie générale n'a été développé jusqu'à présent pour prédire cette réponse et ma thèse montre, qu'en réalité, un telle prédiction est encore plus compliquée parce que la réponse dépend du génotype de la plante considérés, différents génotypes de la même espèces pouvant avoir des réponses contrastées. On ne peut donc pas exclure que l'étude d'une autre plante, le maïs par exemple, dans d'autres sols tropicaux, et en présences d'autres espèces de vers aurait abouti à la même conclusion quand à l'absence de synergie entre les vers et le biochar. En particulier, *P. corethrurus* est une espèce endogée, et une espèce anécique ou la combinaison d'une espèce anécique et d'une espèce endogée aurait pu conduire à des résultats très différents.

Une autre nuance doit être apportée à mes résultats. Il s'agit d'expériences en micro et mésocosmes. Bien sûr l'approche a été fructueuse et l'expérience de 2 ans en mésocosmes confirme les résultats des expériences plus courtes, cependant il n'est pas sûr que la faible taille des microcosmes et des mésocosmes et la faible durée des expériences relativement à la lenteur de certains processus clefs mis en jeux par les expériences (dynamique de la matière organique, dynamique du biochar, dynamique des nutriments minéraux) n'est pas conduit à certains biais. Bien sûr, contrôler la densité de vers aux champs sur le long terme est très difficile mais cela apporterait des renseignements précieux et des méthodes ont étés développée pour répondre à un tel besoin (Bohlen *et al.* 1995).

J'ai en outre montré que différents génotypes de riz ont une capacité de réponse très différente aux vers de terre, au biochar et à la combinaison de des deux. Ces résultats sont à confirmer par des expériences au champ mais ont des conséquences pratiques, pour la gestion soutenable de la fertilité du sol. Dans le cas du biochar cela montre que parallèlement au développement de ''nouvelles'' techniques agricoles comme l'utilisation du biochar il faut développer de nouveaux cultivars qui répondent particulièrement à ces pratiques. Même si les vers de terre sont a priori un facteur naturel des sols, et même si on les retrouve dans les agro-écosystèmes, les gènes permettant aux plantes de répondre positivement aux activités des vers ont pu être perdus stochastiquement au cours de décennies de sélections intensives. Ces gènes ont pu aussi être contre-sélectionnés pour trois raisons: (1) Les sols cultivés pour lesquels les

cultivars modernes ont été développés sont souvent pauvres en vers de terre du fait de pratiques agricoles ''agressives'' (Edwards & Lofty 1982; Mäder *et al.* 2002). (2) Les techniques agricoles modernes sont souvent fondées sur une exploitation du sol et des apports massifs d'engrais plus que sur des rétroactions positives entre plantes, organismes du sol, et sols (Boody & DeVore 2006). (3) Il peut exister des trade-off entre les mécanismes permettant aux plantes de répondre positivement aux vers et les mécanismes leur permettant de répondre, par exemple, à de fortes doses d'engrais minéral. Ma thèse confirme que pour développer des variétés répondant particulièrement bien aux vers de terre, et probablement au biochar il faudrait diversifier les sources de génotypes (variétés) utilisés en sélection et probablement utiliser des variétés anciennes et rustiques voir utiliser des ancêtres non-domestiqués (Boody & DeVore 2006).

La différence de sensibilité de différents génotypes aux vers de terre suggère aussi une conclusion plus théorique. Si la présence de vers change le classement de génotypes, à l'intérieur d'une même espèce, en termes d'accumulation de biomasse mais aussi en termes de nombre de descendants, cela montre que les vers changent la fitness relative de ces génotypes et peuvent donc exercer une pression de sélection sur cette espèce. Bien sûr, les différents génotypes testés ici ont été sélectionnés artificiellement, et leur comportement ne reflète pas forcément bien le comportement de différents génotypes trouvés au sein d'une même population naturelle. Cependant, il est à remarquer que les différences de réponse aux vers observées sont si importantes que même des différences 10 fois moins importantes pourraient conduire à une évolution sensible des traits permettant aux plantes de croitre mieux en présence des vers, sous réserve que l'héritabilité de ces traits soit suffisamment importante. Dans tous les cas, les résultats de ma thèse son un élément de plus venant s'ajouter à des résultats de modélisation (Barot *et al.* 2007b) et des résultats empiriques (Laossi 2009) suggérant déjà la possibilité que les vers de terre peuvent être une force de sélection pour les plantes et que des espèces ou des génotypes de plantes ''lombricophiles'' ont acquis au cours de l'évolution des traits leurs permettant de profiter au mieux des différentes activités de vers de terre. Deux études allant dans le même sens que ma thèse ont déjà été publiées sur la réponse de cultivars à des mycorhizes (Zhu *et al.* 2001) et à des protozoaires (Somasundaram *et al.* 2008). Toutes ces études ouvrent la voie à la sélection e cultivars instaurant des rétroactions positives avec les organismes du sol.

Une question importante se pose alors. Quels traits permettent à certaines plantes (espèces ou génotypes) de répondre positivement aux vers de terre ou au biochar? Pour répondre précisément à cette question il faudrait sans doute comparer plus de génotypes de riz, travailler sur d'autres espèces cultivées, et faire une méta-analyse regroupant toutes les expériences testant les effets des vers sur la croissance des plantes et documentant précisément les traits de ces plantes (ce qui n'est généralement pas fait dans les articles). On peut cependant déjà essayer d'utiliser les connaissances déjà acquises sur les mécanismes d'action des vers et du biochar pour suggérer les types de traits intervenant. Ma thèse à confirmer qu'une part importante de l'action des vers et du biochar passe par l'augmentation de la disponibilité des nutriments minéraux. Des traits permettant aux plantes d'accéder efficacement à ces nutriments libérés par les vers ou retenus par le biochar seront donc favorables. A priori il peut s'agir d'abord de l'architecture du système racinaire et de sa sensibilité à des sources plus ou moins ponctuelles dans le temps et l'espace de nutriments minéraux, i.e. les traits qui définissent les stratégies de ''root foraging'' des plantes (Ingestad & Agren 1991; Bell & Sultan 1999; Farrar *et al.* 2003). Des traits particuliers pourraient aussi être impliqués dans la capacité d'une racine à inverser le processus d'adsorption des nutriments minéraux sur les particules de biochar.

Enfin, mes résultats confirment des résultats plus anciens (Blouin *et al.* 2006) suggérant que les vers agissent aussi par la production de molécules signales dans le sol (Nardi *et al.* 1988; Muscolo *et al.* 1999; Quaggioti *et al.* 2004). Dans ce cas, à un niveau physiologique fin, différents traits doivent être impliqués dans la sensibilité de plantes à des molécules analogues aux phytohormones se trouvant dans le sol: transporteurs racinaires, récepteurs hormonaux ...etc. D'une manière générale, s'il y a bien changement de l'allocation des ressources par une manipulation de types hormonales de vers de terre, tous les gènes intervenant dans cette allocation peuvent intervenir dans la réponse aux vers. D'autre part, si la production de phytohormones passe bien par la stimulation de bactéries du sol, l'action d'une plante (génotype ou espèce) sur les groupes de bactéries impliquées peut être déterminante (Lambers *et al.* 2009): si une plante diminue la biomasse des bactéries de ce groupes, elle sera peut sensible à l'effet ''phytohormonal'' des vers. Du fait que tous ces traits, impliqués dans la réponse aux vers mais aussi au biochar, sont des traits fondamentaux du fonctionnement d'une plante arriver à préciser ces traits et déterminer leur mode d'action ferait certainement faire des progrès importants en physiologie végétale et pourrait déboucher sur des découvertes inattendues.

7.3 Perspectives

La première perspective du travail de thèse présenté dans le présent mémoire serait, comment souvent, de finir les expériences en cours et leur analyse. Il reste d'abord à faire l'analyse statistique des données de l'échantillonnage de terrain visant à tester l'effet à long terme du biochar sur la faune du sol. Dans le même ordre d'idée, les données de l'expérience sur les variétés de riz pourraient être analysées d'une manière plus détaillée. En effet, dans la thèse je ne présente que des résultats portant sur les variables les plus souvent étudiées (biomasse totale, biomasse racinaire, nombre de grains) mais rien sur des variables ''plus fines'' potentiellement intéressantes comme celles issues de l'analyse du système racinaire par scanner et qui sont déjà disponibles. On peut aussi penser qu'étudier les corrélations entre les différentes variables mesurées sur les plants de riz pourrait aider à détecter certains traits liés à la réponse des différents génotypes (voir ci-dessus).

D'autres données ne sont pas encore disponibles mais pourraient provenir d'analyses complémentaires issues de matériel déjà disponible provenant des expériences que j'ai réalisées. Ainsi, l'ARN a déjà été extrait d'échantillons issues des différentes variétés et traitements de l'expérience comparant la réponse de différents cultivars. Il serait intéressant de mesurer sur ces échantillons l'expression de différents gènes de l'anabolisme et du catabolisme comme déjà réalisé sur une des variétés (Chapitre 5). Il serait aussi très utile de mesurer l'expression d'autres gènes: d'autres gènes liés à l'anabolisme de l'azote, mais aussi les gènes liés à la croissance (et architecture) racinaire (Zhang *et al.* 2007) et à l'absorption et assimilation de l'azote sous forme de nitrate (nitrate réductase) ou d'ammonium. Etudier ces derniers gènes permettrait en effet d'aborder les entrées d'azote dans la plante et de tester directement l'action possible de phytohormones. Mesurer l'effet des vers et du biochar sur d'autres gènes pourrait d'une manière générale aider à comprendre quels sont les mécanismes d'action des vers et du biochar et, dans le cas, de l'expérience ''variété'' pourrait aider à détecter les traits physiologiques impliqués dans la réponse positive des plantes.

De la même façon, j'ai gardé des échantillons de sol sec de toutes mes expériences. Ces échantillons pourraient servir à des analyses microbiologiques (biomasse totale, structure de la communauté par DGGE, activité enzymatique ...etc). Les vers (Brown 1995) et le biochar (Pietikäinen & Fritze 2000) modifient à la fois la structure et l'activité des communautés microbiennes. Cela pourrait permettre de tester plusieurs hypothèses. Premièrement, nous avions prévu que les vers et le biochar interagiraient dans leur action sur

la croissance du riz et que cela pourrait se produire parce que, conjointement, ils ont un effet original sur les communautés microbiennes. Même si mes résultats suggèrent que les vers et le biochar n'interagissent pas dans leur action sur les plantes, il serait quand même intéressant de tester s'ils interagissent dans leur effet sur les microorganismes. Deuxièmement, on commence à montrer que différents génotypes de la même espèce de plantes peuvent établir des interactions différentes avec les microorganismes du sol (Lambers *et al.* 2009). Le sol de l'expérience ''variétés'' permettra de tester cette hypothèse chez le riz et en plus de déterminer si la capacité des cultivars de riz à répondre au biochar ou aux vers dépend de leur influence sur les microorganismes du sol.

Ma thèse montre aussi la nécessité de réaliser de nouvelles expériences. Comme expliqué ci-dessus, la réponse des plantes aux vers de terre dépend énormément, et d'une manière assez imprévisible, de la combinaison, espèce de vers-espèces de plante-type de sol. Mon étude est la première à bordé ce type de question pour le biochar. Il faudrait donc répéter le type d'expérience que j'ai mené pour d'autres espèces de plantes, y-compris des plantes non cultivées. Même si ma thèse suggère qu'il n'y a pas de synergie entre biochar et vers de terre, il s'agira bien sûr dans le futur de tester la robustesse de ce résultat, avec d'autres espèces (vers et plantes) et d'autres sols.

Ma thèse ouvre aussi des pistes pour des travaux de plus longue haleine. D'une part elle suggère qu'on pourra dans le futur sélectionner des variétés répondant particulièrement aux vers de terre ou à d'autres groupes d'organismes du sol. Pour arriver à un tel résultat et pousser les spécialistes de la sélection variétale à développer de telles variétés, il faudra d'abord arriver à confirmer qu'une telle approche permet bien d'augmenter l'efficacité de la boucle de rétroaction plantes-organismes du sol et que cela augmente la durabilité de production agricole. Il s'agirait alors d'une agriculture ''intensivement écologique''.

D'autre part, une autre piste ouverte par ma thèse est celle des stratégies métaboliques *K* et *r* par analogie avec deux types de stratégies bio-démographiques mis en évidence en écologie (Pianka 1970). Mes résultats ont montré qu'on pouvait augmenter la production végétale, soit en augmentant à la fois le métabolisme et le catabolisme azoté (plus de protéines créées et plus de protéines dégradées), soit en ne changeant pas ce métabolisme. Il faudrait tester si ce type de résultats est généralisable à d'autres plantes et d'autres facteurs environnementaux. On voit alors se dessiner l'esquisse d'une possible interaction féconde entre écologie et physiologie moléculaires des plantes. D'une part on pourrait classer et

ordonner des génotypes, des espèces ou des facteurs environnementaux sur le continuum *r* – *K*. A l'inverse, étudier finement la physiologie des plantes pourrait permettre de mettre en évidence et de décrire précisément les trade-off (compromis) qui sont à la base de nombreuses théories de l'écologie et de l'écologie évolutive. De tels trade-off sont en effet très difficile à mettre en évidence avec des variables macroscopiques et pourraient être plus facile à étudier sur le plan des mécanismes métaboliques comme cela a pu être fait en microbiologie (Velicer & Lenski 1999; Pfeiffer *et al.* 2001) .

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ANNEXE

9 Annexe

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Effects of an endogeic and an anecic earthworm on the competition between four annual plants and their relative fecundity

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ABSTRACT

Competition between plants for essential resources determines the distribution of biomasses between species as well as the composition of plant communities through effects on species reproductive potentials. Soil organisms influence plant competitive ability and access to resources; thus they should modify plant community composition. The effects of an endogeic (*Aporrectodea caliginosa*) and an anecic (*Lumbricus terrestris*) earthworm species on the competition between grass (*Poa annua*), two forbs (*Veronica persica and Cerastium glomeratum*) and legume (*Trifolium dubium*) were investigated in a greenhouse experiment. We established two types of plant communities: monocultures and polycultures of the four species. *L. terrestris* increased the biomass of *P. annua* and *V. persica* (in monocultures as well as in polycultures). However, the presence of *L. terrestris* allowed the grass to produce the highest biomass in polycultures suggesting that this earthworm species promoted the growth of *P. annua* against the other plant species. In monocultures as well as in polycultures, the presence of *L. terrestris* to increased the number of seeds of *T. dubium* and the total seed mass of *V. persica*. These results suggest that *L. terrestris* enhanced the short term competitive ability of *P. annua* by promoting its growth. The increased number of seeds of *T. dubium* in the presence of *L. terrestris* suggests that this earthworm species could enhance the long-term competitive ability of this legume and may increase its number of individuals after several generations.

1. Introduction

Soil organisms are known to affect plant growth by enhancing mineralization of soil organic matter, modifying soil physical and chemical properties, consuming plant roots or maintaining symbiotic and parasitic relations with plants (Lavelle and Spain, 2001; Wardle et al., 2004; Bardgett, 2005). Many studies have been published on this topic. Most of them examine effects of soils organisms on plant growth, are short term microcosm experiments that focus on plant monocultures (Scheu, 2003). However, over a longer period, during an entire generation, soil organisms may also influence plant survival and fecundity (Poveda et al., 2005a,b). Moreover, few experiments have determined the effect of soil organisms on plant communities and compare the response of plant species when grown in monocultures and in polycultures (Bliss et al., 2002; Bonkowski and Roy, 2005; Eisenhauer et al., 2008a,b). Since these responses might be different, soil organisms may change the relative competitive ability of plant species and not only their growth and reproductive potential in monocultures (Bever, 2003; Reynolds et al., 2003; Wurst et al., 2004; Eisenhauer et al., 2008a,b). Both interspecific competition and soil organisms are likely to change interactively the plant hierarchy in growth, survival and reproductive ability. This may simply occur because a small initial advantage in their growth allows them to capture a higher proportion of resources (Weiner, 1990). It may also occur when soil organisms release mineral nutrients that benefit all plant species when grown in monocultures, but mostly benefit the species that are more efficient at absorbing these nutrients in polycultures. Taken together, the comparison of soil organism effects on the growth and reproduction of different plant species in monocultures and polycultures is necessary to predict the long-term effect of soil organisms on plant communities.

Among soil organisms, earthworms are known to generally affect positively plant (Scheu, 2003; Brown et al., 2004). They are also known to affect seed germination (Grant, 1983; Decaëns et al., 2003; Milcu et al., 2006). However, few studies have investigated their effects on plant competition and plant community structure –

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as compared to the abundance of studies on single plant species (Scheu, 2003; Brown et al., 2004). To our knowledge, none has tested the effects of earthworms on plant competition taking into account the whole plant life-cycle (from germination to seed production). Furthermore, despite the evidence that different functional groups of earthworms can differentially affect plant growth (Lavelle et al., 1998), no study has tested for the effect of earthworm species belonging to different functional groups and their interaction on plant performance in the same laboratory experiment.

We performed a microcosm experiment and we evaluated the effects of an anecic and an endogeic earthworm species on seed germination, plant growth and seed production in four annual plant species growing in monocultures (intraspecific competition) or polycultures (interspecific competition). Endogeic earthworms keep moving inside the soil to feed on soil organic matter while anecic feed on plant litter at the soil surface and tend to stay in the same burrow (Lavelle et al., 1998). Anecic earthworms fragment plant litter and incorporate it into the soil where it can subsequently be ingested by endogeic earthworms. Such an interaction can lead to higher mineralization and plant growth (Jégou et al., 1998; Brown et al., 2000). We hypothesized that different plant species belonging to different functional groups should be affected differently by earthworms (Eisenhauer et al., 2008a,b). For example, legumes are supposed to be relatively insensitive to earthworm effects via an acceleration of mineralization since they have a direct access to atmospheric nitrogen (Brown et al., 2004). Specifically we tested four hypotheses: (1) earthworms change plant relative competitive ability in term of growth; (2) earthworms also influence plant relative reproductive potentials; (3) *Aporrectodea caliginosa* (endogeic earthworm) and *Lumbricus terrestris* (anecic earthworm) affect differently plant competition; (4) there is an interactive effect between *A. caliginosa* (an endogeic species) and *L. terrestris* (an anecic species) on plant growth and reproductive potential.

2. Materials and methods

2.1. Experiment set up

Experiment containers (microcosms) consisted of PVC pots (diameter 18 cm, height 17 cm). Drains at the bottom of pots were covered with 1 mm plastic mesh to prevent earthworms from escaping. Soil was collected at the ecology station of the Ecole Normale Supérieure at Foljuif (France). It is a sandy cambisol supporting a meadow (OM = 2.55%, C/N = 12.4, C content 1.47%, Ntotal = 0.12%, pH = 5.22). A total of 100 microcosms were filled with 3 kg of sieved (2 mm) dry soil. Before starting our experiment, the microcosms were watered regularly for two weeks and germinating weeds from the seedbank were removed. Prior to the addition of earthworms and seeds, 8 g of dried litter (72 h at 60 °C) of grass leaves were placed at the soil surface and 1 g was mixed with the first centimeter of soil. This constituted the essential food resource for the anecic earthworm species.

We used an anecic earthworm, *L. terrestris* (L.) (LT), and an endogeic earthworm, *A. caliginosa* (Savigny) (AC). These earthworm species are among the most abundant in temperate ecosystems (Edwards and Bohlen, 1996; Bohlen et al., 2004). LT was purchased in a store and AC was collected in the park of the IRD centre in Bondy (France). Our experiment had three earthworm treatments (AC, LT, AC + LT) and a control without earthworm. Five replicates were implemented for each treatment combination, resulting in 20 microcosms for the 4 earthworm treatments and for each plant treatment (see below). One individual of LT (4.2 ± 0.5 g) and four of AC (2.8 ± 0.4 g, i.e. total biomass of 4 AC individuals with gut contents) were introduced in each treatment including these species. The biomass of specimens added was equivalent to 165 g/m^2 and 110 g/m^2 for LT and AC respectively, which is comparable to the biomasses found in grassland ecosystems (Edwards and Bohlen, 1996).

Five days after introducing earthworms, 20 seeds of *Veronica persica*, *Trifolium dubium*, *Cerastium glomeratum* or/and *Poa annua* were sown, either in monocultures (4 × 20 microcosms) or in polycultures of the four species (20 microcosms). The seed size of the plant species were respectively 0.4 × 0.6 mm (±0.08) for *C. glomeratum*, 1.0 × 1.1 mm (±0.2) in *V. persica*, 1.1 × 1.3 mm (±0.1) in *T. dubium* and 1.9 × 1 mm (±0.1) in *P. annua* (obtained by measurement of 20 seeds of each plant species). Three weeks later, seedlings of each monoculture were counted to determine the germination rate. Four plants per microcosm (4 plants of the same species in monocultures, one plant of each species in polycultures) were kept (other seedlings were removed). Microcosms were weeded weekly during the experiment. Microcosms were watered during 7 weeks with 12.5 ml and from the eighth week to the end (week 15) with 25 ml each day. This allowed us to maintain the soil near its field capacity (this was checked through regular weighing of some pots).

2.2. Sampling

Seeds were harvested from plants as they matured. On week 15, shoots of the four species were cut at the soil surface and dried separately at 60 °C for 72 h. Roots were separated from soil by washing on a 600 µm mesh, but the roots of the individual plant species were not recognizable in polycultures. Individual dried shoot biomass and total root biomass were weighed. Twenty seeds from each plant were randomly selected and weighed as well as the biomass of all seeds. These data were used to calculate the number of seeds per plant and mean seed mass. 95% of the earthworms were recovered at the end of the experiment (77% of the total mortality was due to *A. caliginosa* [10 individuals] and 27% to *L. errestris* [3 individuals]). N concentration in plant leaves was measured using a ThermoFinnigan Flash EA 1112 elemental analyzer (ThermoFinnigan, Milan, Italy).

Table 1
General ANOVA table forthe effects of earthworms (AC and LT), plant species and composition (monocultures or mixtures) on shoot biomass, number of seeds, total seed mass and mean seed mass. *F*-values and the corresponding *p*-values are displayed. Data on seeds were log transformed.

	df	Shoot biomass	Number of seeds	Total seed mass	Mean seed mass
		F	*F*	*F*	*F*
AC	1	1.94	0.79	0.47	0.00
LT	1	10.66**	1.30	1.78	0.09
Composition	1	63.07***	3.81*	35.39***	103.46***
Plant species	3	183.72***	39.77**	77.19***	6.86***
AC × LT	1	1.73	0.00	0.03	0.04
LT × plant species	3	12.46***	2.23+	4.40**	4.41**
AC × plant species	3	0.65	1.62	1.98	0.87
AC × composition	1	0.08	0.42	0.29	0.05
LT × composition	1	3.28	0.77	0.11	0.23
Composition × plant species	3	29.71***	1.28	2.70	0.57
AC × LT × plant species	3	0.37	2.29+	2.25	1.70
AC × LT × composition	1	0.00	0.02	0.20	0.90
AC × plant species × composition	3	0.50	0.57	1.65	0.68
LT × plant species × composition	3	6.07***	0.23	0.14	0.33
AC × LT × plant species × composition	3	2.04	0.14	0.11	0.82
r^2		0.87	0.62	0.77	0.64

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; +$p < 0.1$.

Table 2
ANOVA table for the effects of earthworms (AC and LT) and plant species on the number of seedlings (seed germination), root biomass, total specific biomass and shoot-to-root ratio in monoculture. *F*-values and the corresponding *p*-values are displayed. Total df = 79.

	df	Number of seedlings	Root biomass	Total biomass	Shoot-to-root ratio
		F	*F*	*F*	*F*
AC	1	0.12	0.49	2.07	0.56
LT	1	60.38***	3.50*	4.32*	0.56
AC × LT	1	0.01	0.35	2.70	0.37
Plant species	3	107.81***	97.43***	139.40***	17.99***
LT × plant species	3	4.36**	3.20*	3.77**	1.20
AC × plant species	3	0.66	0.23	0.13	0.34
AC × LT × plant species	3	0.34	0.67	2.27	0.48
r^2		0.86	0.83	0.87	0.49

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2.3. Statistical analyses

Data were analysed with ANOVAs using SAS GLM procedure (Sum of squares type III, SS3) (SAS, 1990). A full model was used to test all possible factors ("AC", "LT", "plant species" and "composition" i.e. monocultures or polycultures) and all interactions between these factors (Table 1). The factor "composition" indicates whether plant species are grown in polycultures or monocultures. Testing the interaction between this factor and earthworm effects allowed us to determine whether earthworm effects on plant depend on the nature of competition (intraspecific vs. interspecific). Similarly, testing the interaction between plant species and earthworm effects allowed us to determine whether plant species responded differently to earthworm species. Since roots of the four species were not distinguished in polycultures, analyses of specific root biomass and specific total biomass (shoot + root) were only achieved on monocultures (Table 2). Seed germination was also only analysed in monocultures (Table 2). In these analyses, biomasses from monocultures were divided by 4 to allow comparing monocultures and polycultures (since in monocultures we had 4 individuals of the same species while in polycultures we had one individual of each plant species). To determine the direction of significant effects, we used multiple comparison tests based on least square means (LSmeans, LSmeans SAS statement) but we only present the general outcome of these comparisons without displaying them in detail. The residuals of each model were analysed to test for normality and homogeneity of variances. Logarithm transformation was used for the number of seeds and seed mass. All tests were achieved with a significance level $\alpha = 0.05$.

3. Results

The statistical analysis (Table 1) showed that LT differently affected intra- and interspecific competition between the different plant species (significant LT × plant species × composition interaction). Reproductive parameters of the plant species, such as seed number and seed biomass were also differently affected by LT ($p < 0.1$). Earthworms did not affect the shoot/root ratio of any plant species and no simple effect of AC on plants was found.

At the end of the experiment we found a decline in earthworm biomass per microcosm when compared to the initial biomass, average final biomass of *L. terrestris* 2.62 g (−48%) and *A. caliginosa* 1.39 g (−51%). This is probably due to the low organic matter concentration of the soil in the case of *A. caliginosa*, and to the total disappearance of plant litter before the end of the experiment in the case of *L. terrestris*. We checked that earthworm biomass (initial

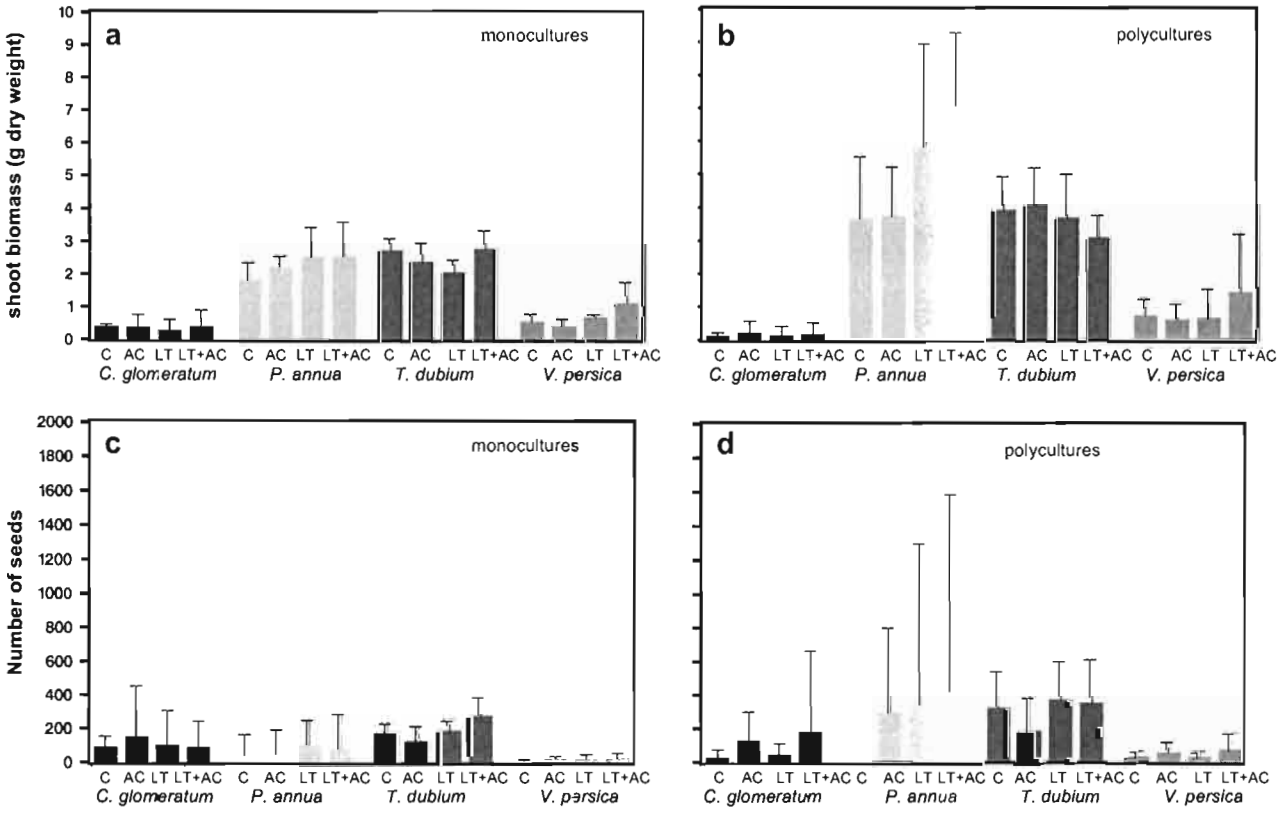

Fig. 1. Effects of earthworms on shoot biomass in monocultures (a) and polycultures (b), and on the number of seeds in monocultures (c) and in polycultures (d). C, control treatment; AC, *A. caliginosa* only; LT, *L. terrestris* only; LT + AC, combined treatment with *A. caliginosa* and *L. terrestris*. Means are displayed together with SD.

biomass as well as final biomass) did not affect plant performance ($p > 0.05$) so that earthworm biomasses were not taken into account in the other analyses.

The presence of LT increased the shoot biomass of *P. annua* (+36%) and *V. persica* (+80%). In *V. persica* the highest shoot biomass was obtained in the treatment with both earthworm species (Fig. 1a and b). Shoot biomasses of *T. dubium* and *C. glomeratum* were not affected by *L. terrestris* (Fig. 1a and b). Differences in shoot biomass between monoculture and polycultures were found for all plant species except *V. persica*. *P. annua* and *T. dubium* produced higher shoot biomass in polycultures than in monocultures, demonstrating that these species gained relative dominance in interspecific competition; while the opposite was found for *C. glomeratum*, which had a higher shoot biomass in monoculture (see Fig. 1a and b). The effects of the community composition on the shoot biomass of *P. annua*, however, depended on the presence of LT (Table 1, significant LT × plant species × composition interaction). The highest shoot biomass was found for *P. annua* in polycultures and in the presence of LT (detailed results not presented but see Fig. 1b). Its contribution to the community aboveground biomass increased in presence of the anecic earthworm (Fig. 3a).

The presence of LT increased the number of seeds per *T. dubium* individual by 60%. Also the highest number of seeds per plant was obtained in the treatment with both earthworm species. The presence of LT decreased (−42%) the mean seed mass of *T. dubium* (indicating that plants produced more but smaller seeds) and increased (+60%) the total seed mass of *V. persica* (LSmeans comparisons and significant LT × plant species interaction in Table 1). *T. dubium* and *V. persica* produced higher number of seeds in polycultures while the opposite was found for *C. glomeratum* (Fig. 1c and d). The total seed mass of *V. persica* (2.5 times) and *C. glomeratum* (5 times) were higher in monocultures than in polycultures and the mean seed mass of *C. glomeratum* was four times higher in monocultures.

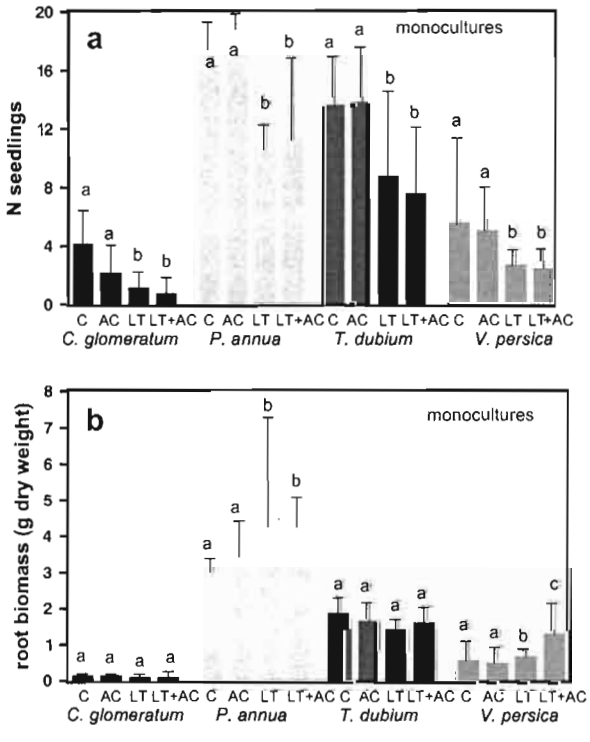

Fig. 2. Effects of earthworms on (a) the number of seedlings in monocultures, (b) root biomass in monocultures. C, control treatment; AC, *A. caliginosa* only; LT, *L. terrestris* only; LT + AC, combined treatment with *A. caliginosa* and *L. terrestris*. Bars with different letters are significantly different at $p < 0.05$ according to LSmeans comparisons. Means are displayed together with SD.

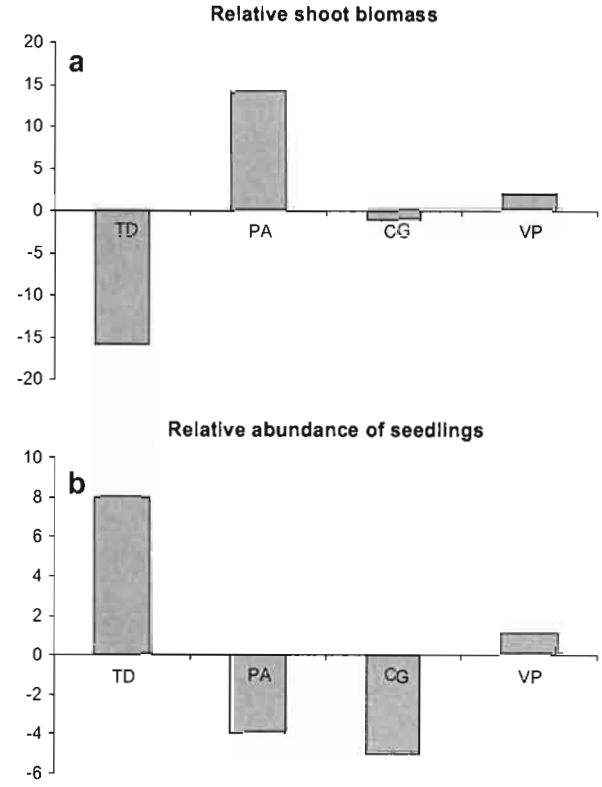

Fig. 3. Effect of the presence of LT on relative shoot biomass of each species (a) in the plant community calculated as [% in shoot biomass of the community; in presence of LT] − [% in shoot biomass of the community; in absence of LT] and (b) on the relative abundance of plant species in the hypothetical second generation community calculated as [% seedlings, i.e. germination × number of seeds; in presence of LT] − [% seedlings, i.e. germination × number of seeds; in absence of LT]. TD, *Trifolium dubium*; PA, *Poa annua*; CG, *Cerastium glomeratum*; VP, *Veronica persica*.

The presence of LT decreased the number of seedlings for all plant species in monocultures. However, plant species were affected differently (Fig. 2a and significant interaction LT × plant species in Table 2). The presence of LT decreased less the seed germination of *T. dubium* (−59%) and *P. annua* (−44.5%) than for *V. persica* (−88%) and *C. glomeratum* (−95%). LT increased the root biomass (+33%) and the total biomass (+27%) of *P. annua* while it did not affect the root and the total biomass of the other plant species (Table 2 and LSmeans comparisons).

Earthworm presence affected the nitrogen concentration in plant leaves (Table 3 and Fig. 4). LT activity increased the N content of *P. annua* (+14%) but decreased N content of *V. persica* (−25%). The presence of AC increased the N content in *C. glomeratum* (+44%) and *V. persica* (+33%).

Table 3
ANOVA table for the effects of earthworms (AC and LT) on the nitrogen content in plant leaves in mixture. *F*-values and the corresponding *p*-values are displayed. Total df = 19.

	df	*T. dubium*	*P. annua*	*C. glomeratum*	*V. persica*
		F	*F*	*F*	*F*
AC	1	0.78	3.29	19.02***	43.52
LT	1	3.72	4.91*	1.68	51.47***
AC × LT	1	1.48	0.34	14.21**	69.55***
r^2		0.26	0.34	0.68	0.92

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

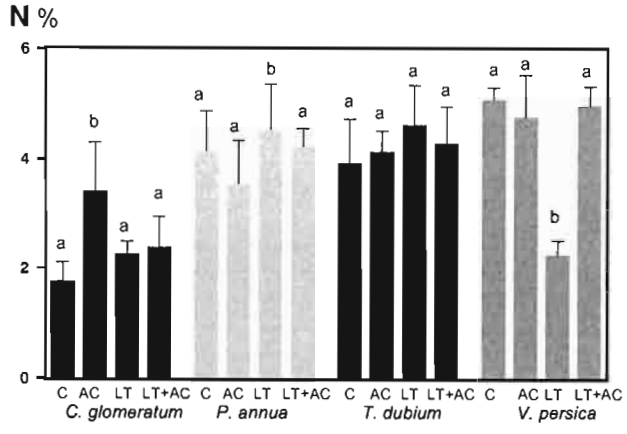

Fig. 4. Effects of earthworms on N concentration in leaves of *Trifolium dubium*; *Poa annua*; *Cerastium glomeratum* and *Veranica persica*. Bars with different letters are significantly different at $p < 0.05$ according to LSmeans comparisons. Means are displayed together with SD.

4. Discussion

Taking into account the whole life-cycle of four annual plants, we have found that (1) LT decreased less the germination of *T. dubium* and *P. annua* relative to the two other plant species. (2) LT increased the biomass production and the nitrogen concentration in *P. annua* relatively to the three other plants species. (3) LT increased the production of seeds by *T. dubium* relative to the three other plant species. (4) The presence of AC and its interaction with LT (AC × LT) increased the nitrogen concentration of the aerial system of *C. glomeratum* and *V. persica*. However, despite this effect, AC did not affect the growth and seed production of the four plant species.

4.1. Earthworm effects on plant competition

Our findings on seed germination are consistent with those of Milcu et al. (2006) showing that LT reduced strongly the germination rate of small seeds such as the seeds of *Poa pratensis*, *Trifolium repens*, *Bellis perennis* and *Festuca pratensis*. Such a decrease in the germination rate might either be due to seed burrowing by earthworms or to the fact that seeds are damaged during their ingestions by earthworms (Mc Rill and Sagar, 1979; Grant, 1983). Besides, the main positive effects of earthworms on plant growth found in our study are consistent with others showing that earthworms enhance the growth of grasses while legumes barely responded to earthworms (Wurst et al., 2003, 2008; Brown et al., 2004; Eisenhauer et al., 2008a,b). Since legumes fix atmospheric nitrogen through their association with *Rhizobium*, they are more independent than other plants from the availability of soil nitrogen. The small effect of the anecic earthworm on *P. annua* shoot biomass in monoculture was amplified when the grass was in mixture probably as a result of interspecific competition: in competition with other plant species *P. annua* is likely to have absorbed a greater share of the nutrient made available by LT than the other plant species. This argument is supported by the fact that LT increased N concentration in *P. annua* leaves. The increased seed production of *T. dubium* in the presence of LT could be an indirect result of a stimulation of symbiotic fixation by earthworms which has already been pointed out (Doube et al., 1994). It is less clear why *C. glomeratum* and *V. persica* did not react to the likely increase in mineral nutrient availability in presence of LT either in monoculture or mixture.

The positive effect of an earthworm species on the production of seeds has previously been reported by Poveda et al. (2005a,b) for wild mustard. However, the effect of earthworms on plant fecundity (and not only the seed biomass) has rarely been examined. In our case, LT did not increase the biomass of whole *T. dubium* individuals but enhanced the productions of seeds (+60%) that were smaller. This shows that LT changes the resource allocation of *T. dubium* in such a way that it increased its fecundity. This result might explain why the studies reporting a promotion of clover by earthworms were generally long-term (more than 9 months and at least two plants generations) experiments (Hopp and Slatter, 1948; Thompson et al., 1993) while short term experiments usually show very small effects of earthworms on the biomass of legumes (Brown et al., 2004; Kreuzer et al., 2004).

Contrary to many former results (Wurst et al., 2003, 2005; Kreuzer et al., 2004), no effect of AC and few effects of the interaction between AC and LT were found on plant growth and reproduction in this study. This general pattern could be due to the low content of the soil in organic matter. Indeed, in such a soil endogeic earthworms do not necessarily promote plant growth because there is little organic matter to be mineralized, thus few nutrients are released. However, AC increased the N content of the aerial system of *C. glomeratum* and *V. persica*. This suggests that AC can influence the physiology of these plants, for example the allocation of N to the root and aerial systems without affecting their biomass. Up to our knowledge, despite the fact that plant nutrient allocation has been widely studied (Lambers et al., 1998), predicting such effects of soil organisms on this allocation and determining the involved mechanisms remains very difficult.

Taking into account LT effect on seed germination and on seed production in our experiment we can theoretically estimate that, in similar conditions, this earthworm species should increase, at least at an early stage of the second generation, the abundances of *T. dubium* and *V. persica* seedlings in the community but decrease the abundances of *C. glomeratum* and *P. annua* (see Fig. 3a). These results contrast with LT effect on the contribution of each species to the shoot biomass of the whole community (+14.8% for *P. annua*, −15% for *T. dubium*, Fig. 3a; comparison between Fig. 3a and b). Hence, earthworm could promote one species at the individual scale, on a short time scale (a generation), but disadvantage this species at the community scale, on a longer time scale (across-generations) and vice versa. We have thus pointed out a potentially important mechanism but making predictions on the long-term effect of earthworms on plant community structure in the field would require taking into account many other processes, for example the effects of other organisms such as herbivores which can alter plant responses to earthworms (Poveda et al., 2005a,b).

4.2. Plant community type effects on plant growth

Plants responded differently when grown in monocultures and polycultures. Polycultures had a higher total biomass than monocultures (detailed statistical analysis not displayed in the results section). This suggests that the four species communities were dominated by the effect of complementary resource use, which decreased the negative impact of interspecific competition. More specifically, both *T. dubium* and *P. annua* produced higher shoot biomasses by individual in polycultures, which suggests that their relation is driven by complementary resource use. This could be explained by the fact that grasses are generally limited by N and legumes by P (Hooper, 1998). On the contrary, when *T. dubium* and *P. annua* were grown with *C. glomeratum*; the shoot biomass of the later decreased. This suggests, that *C. glomeratum* is a poorer competitor than the two other species, and that its relation with these species is driven by the competition for common resources. In the same vein, *T. dubium* and *C. glomeratum* individuals produced respectively more and less seeds and shoot biomass in interspecific than in intraspecific competition. This is probably merely

a consequence of the respectively high and low abilities of these two species to capture resources, and thus of their short term competitive abilities. On the contrary, interspecific competition did not significantly change the biomass of *V. persica* but increased the number of seeds produced by individual and decreased the total seed biomass (i.e. interspecific competition led to a shift in the resource allocation). This should increase the number of *V. persica* individuals within the community in the next generation and thus could enhance its across-generation competitive ability. Up to our knowledge such a response to interspecific competition has never been pointed out, and very few studies have tackled the issue (Aarssen and Keogh, 2002).

4.3. Conclusions

Through its effect on germination and seed production LT is likely to modify the demography of the different plant species and to change the relative abundance of the plant species in the community after several generations. Earthworms can thus be considered to modulate long-term competition between plants. These results show that belowground–aboveground interactions have not only short term effects on plant growth (Wilson et al., 2001). They should also have demographic consequences that have so far been seldom studied. This emphasizes the importance of studying the effects of belowground–aboveground interactions on the whole life-cycles of plants, because first these effects might differ at different stages of the life-cycle and second to predict their consequences on plant demography and plant community structure (Wurst et al., 2008). In our case, it still remains to study the effect of earthworms on plant survival after the seedling stage. The effect of soil organisms on biomass production has often been documented, however, their effects on resource allocation are seldom documented, and there is so far no theory to predict how plant species shift their resource allocation strategies. For example the percentage of biomass and nitrogen allocated to seeds and the size of each seed, in the presence of different soil organisms must be investigated. Such a theory is nevertheless required to predict plant population or plant community responses to soil organisms.

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REGULAR ARTICLE

Earthworm effects on plant growth do not necessarily decrease with soil fertility

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Abstract Earthworms are known to generally increase plant growth. However, because plant-earthworm interactions are potentially mediated by soil characteristics the response of plants to earthworms should depend on the soil type. In a greenhouse microcosm experiment, the responsiveness of plants (*Veronica persica, Trifolium dubium and Poa annua*) to two earthworm species (in combination or not) belonging to different functional groups (*Aporrectodea. caliginosa* an endogeic species, *Lumbricus terrestris* an anecic species) was measured in term of biomass accumulation. This responsiveness was compared in two soils (nutrient rich and nutrient poor) and two mineral fertilization treatments (with and without). The main significant effects on plant growth were due to the anecic earthworm species. *L. terrestris* increased the shoot biomass and the total biomass of *T. dubium* only in the rich soil. It increased also the total biomass of *P. annua* without mineral fertilization but had the opposite effect with fertilization. Mineral fertilization, in the presence of *L. terrestris*, also reduced the total biomass of *V. persica*. *L. terrestris* did not only affect plant growth. In *P. annua* and *V. persica A. caliginosa* and *L. terrestris* also affected the shoot/root ratio and this effect depended on soil type. Finally, few significant interactions were found between the anecic and the endogeic earthworms and these interactions did not depend on the soil type. A general idea would be that earthworms mostly increase plant growth through the enhancement of mineralization and that earthworm effects should decrease in nutrient-rich soils or with mineral fertilization. However, our results show that this view does not hold and that other mechanisms are influential.

Keywords Earthworms · *L. terrestris* · *A. caliginosa* · Plant growth · Soil type · Nutrient availability · Shoot/root ratio

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Introduction

Soil organisms are known to affect plant growth by enhancing mineralisation of soil organic matter and modifying physical and chemical properties of soil

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(Bardgett et al. 2005; Lavelle and Spain 2001). Within soil organisms, earthworms are in term of biomass and activity among the most important detritivores in terrestrial ecosystems (Edwards 2004). They are also known to affect plant growth, generally positively, via five main mechanisms (Brown et al. 2004; Scheu 2003): (1) an increased mineralization of soil organic matter (2) the production of plant growth substances via the stimulation of microbial activity; (3) the control of pests and parasites; (4) the stimulation of symbionts and (5) modifications of soil porosity and aggregation, which induces changes in water and oxygen availability to plant roots. Although these mechanisms are well identified it is difficult to determine their relative influence (Blouin et al. 2006) either in precise cases or in broad classes of cases as defined by plant functional type, geographic area or soil type.

Brown et al. (2004) has remarked that the response of plants to earthworms should depend on the type of soil and especially its texture and its richness in mineral nutrients and organic matter. Indeed, mechanisms through which earthworms influence plant growth might be either down or up-regulated by soil characteristics. For example, if the positive effect of an earthworm species on plant growth is mainly due to an increase in mineralization, the species might no longer increase plant growth in a soil where nutrients are not limiting. However, few studies (Doube et al. 1997; Wurst and Jones 2003) have tested the effect of earthworms on plant in different soils in the same laboratory experiment. Doube et al. (1997) have shown that *Aporrectodea trapezoides* increased the growth of wheat in a sandy soil but not in a clayey one. They also showed that the growth and the grain yield of barley were increased by *Aporrectodea trapezoides* and *Aporrectodea rosea* (both are endogeic earthworms) in the sandy soil but reduced in the clayey. On the contrary, Wurst and Jones (2003) have shown that *Aporrectodea caliginosa* increased the root biomass of *Cardamine hirsute* in two different soils. Up to our knowledge, no laboratory experiment has so far compared the effect of two earthworm species belonging to different functional groups on the same plant species, in soils differing by their texture and nutrient content. Our experiment aims at meeting this need.

Besides, plant species of different functional groups should respond differently to earthworms (Brown et al. 2004) because they are not limited by the same resources and do not have the same resource allocation strategies (Laossi et al. 2009). Legumes, for example, are thought to be less responsive to earthworms than grasses since they are not limited by nitrogen (Brown et al. 2004; Wurst et al. 2005). Finally, plant responses may also depend on earthworm functional group since earthworm belonging to different functional groups differ in their behaviors (Lavelle and Spain 2001). For example, endogeic earthworms keep moving inside the soil to feed on soil organic matter while anecic feed on plant litter at the soil surface and tend to stay in the same burrow (Lavelle et al. 1998). Anecic earthworms fragment plant litter and incorporate it into the soil where it can subsequently be ingested by endogeic earthworms. Such an interaction could increase further mineralization and plant growth (Brown et al. 2000; Jégou et al. 1998).

We tested how the effects of earthworms belonging to different functional groups on plant growth covary with plant functional group and soil type. Hence, we investigated in a microcosm greenhouse experiment the responsiveness of three annual plant species of different functional groups (*Poa annua*, a grass; *Trifolium dubium*, a legume; *Veronica persica*, a forb) to an endogeic (*Aporrectodea caliginosa*) and anecic (*Lumbricus terrestris*) earthworm species as well as to the combination of the two species. The responsiveness of plant was measured in term of biomass accumulation and was compared in two soils (a clayey nutrient rich soil with higher organic matter content and a sandy nutrient poor soil with lower organic matter content) and two mineral fertilization treatments (with and without). Mineral fertilization can be considered either to mimic richer soils or agricultural practices.

We hypothesized that earthworms affected plant growth mainly through an enhancement of mineralization, which is the more often cited mechanism (Kreuzer et al. 2004; Partsch et al. 2006; Wurst et al. 2003). Therefore (see above), both earthworm species should affect (1) plant growth and (2) plant resource allocation only in the soil that is poor in organic matter and mineral nutrient. Similarly, (3) significant interactive effects of the two earthworm species on plant growth and resource allocation should only be found in the poor soil. Finally, according to the same assumption that earthworm mostly influence plants by increasing the availability of mineral nutrients, (4) the

impact of *A. caliginosa* and *L. terrestris* on plants should decrease with mineral fertilization.

Materials and methods

Experiment set up

The experiment was set up in microcosms consisting of PVC pots (inner diameter 14 cm, height 12.5 cm) that were closed at the bottom with 1 mm plastic mesh to prevent earthworms from escaping. A total of 320 microcosms were filled with 950 g (±20 g) of sieved (2 mm) dry soil in a greenhouse. Before starting our experiment, the microcosms were watered regularly for 2 weeks and germinating weeds from the seedbank were removed. Eight grams of dried litter (72 h at 60°C) of grass leaves were placed at the soil surface and 1 g was mixed with the first cm of soil, prior to the addition of earthworms and seeds. This constituted the essential food resource for the anecic earthworm species.

Soils

We used two different soils: A sandy cambisol (called hereafter in the text the "nutrient poor soil") supporting a meadow (OM=2.55%; C/N ratio=12.4; total carbon content=1.47%; Ntotal=0.12%; pH=5.22) collected at the ecology station of the Ecole Normale Supérieure at Foljuif (France) and a clayey leptosol (OM=9.81%; C/N ratio=12.2; total carbon content=5,67; Ntotal=0.465; pH=7.45) collected at the ecology station of Brunoy (France) (called hereafter the "nutrient rich soil").

Earthworms

We used an anecic earthworm, *Lumbricus terrestris* (L.) -LT-, and an endogeic earthworm, *Aporrectodea caliginosa* (Savigny) -AC-. LT were purchased in a store and AC were collected in the park of the IRD centre in Bondy (France). Our experiment consisted in four treatments: AC, LT, AC + LT and a control without any earthworm species (C). One adult of LT (4.2±0.5 g) and three adults of AC (2.4±0.4 g) were introduced in each treatment including these species. This represents respectively 273 g m^{-2} and 156 g m^{-2}, which is comparable to the biomasses found in temperate grassland ecosystems (Edwards and Bohlen 1996). In the treatment with both earthworm species (AC + LT) we have maintained for each earthworm species the biomass used in AC and LT treatment. This was done to maintain the same activity level of each earthworm species, which was the only way to allow testing for a possible interactive effect of the two earthworm species on plant growth. 96% of the earthworms were recovered at the end of the experiment (471 *A. caliginosa* and 143 *L. terrestris* individuals among the 480 and 160 that were originally introduced respectively).

Plants

One week after introducing earthworms, 15 seeds of *Veronica persica, Trifolium dubium* and *Poa annua* were sown in monocultures. Three weeks later, a single plant per microcosm was kept (the other seedlings were removed and cut down in the original microcosm). Microcosms were weeded every week during the experiment. Microcosms were watered during 7 weeks with 6.5 ml every day and from 8th week to the end (week 16) with 13 ml every day. This allowed us to maintain the soil near its field capacity (this was checked through regular weighing of some pots). Microcosm position within the greenhouse was randomized every 2 weeks.

Fertilizer

For each combination of treatments (soil type × LT × AC × plant species), two fertilization treatments were implemented: without or with mineral fertilization. This treatment consists in an application of fertilizer containing N, P, K, S and Mg. 0.6 g of fertilizer was placed at the soil surface at the beginning of the experiment, 0.6 g 3 weeks after sowing and 0.6 g on week 6, when the first flowers were produced. From week 6 to week 12, 1 g of fertilizer was added every 2 weeks before watering. A total of 5.8 g of fertilizer was then added per pot. This corresponds to 48 kg of N and K; 32 kg of P; 6.7 kg of S and 97 kg of Mg per hectare. Five replicates of each treatment combination, i.e. fertilization × soil type × LT × AC × plant species, were implemented.

Sampling

Plants were harvested on week 16. Shoot (leaves and stems) were cut at the soil surface and roots were separated from the soil by washing on a 600 µm mesh.

Root and shoot biomasses were dried at 60°C for 72 h. Dried shoot and root biomasses were weighted. Because of differences in the timing of seed maturation between plant species, seeds were not harvested.

Statistical analyses

Data were analysed with ANOVAs using SAS GLM procedure (Sum of squares type III, SS3) (SAS 1990). A full model was first used to test all factors ("AC", "LT", "plant species", "fertilizer" and "soil") and all interactions between them (Table 1). When significant interactions between plant species and other factors (AC, LT, soil and fertilization) were detected, data were reanalysed separately for each plant species (Table 2) to describe in a more detailed way the effects of these treatments on each plant species. This allowed for example determining which plant species responded to which earthworm species and in which conditions (soil type and fertilization). Effects of treatments and interactions between treatments were tested on shoot biomass, root biomass, total biomass, and shoot/root ratio.

The residuals of each model were analysed to test for normality and homogeneity of variances. To determine the direction of significant effects, we used multiple comparison tests based of least square means taking into account Bonferroni's correction (LSmeans, LSmeans SAS statement). All tests were achieved with a significance level $\alpha=0.05$.

Results

In the present experiment, the main effects are due to the anecic earthworm species, *L. terrestris*. No significant effect of *A. caliginosa* was found. The full statistical model (Table 1) showed that *L. terrestris*

Table 1 ANOVA table of *F*-values for the effects of earthworms (AC and LT), soils, fertilizer and plant species on root, shoot and total biomass and shoot/root ratio (Total df=209)

		Root biomass	Shoot biomass	Total biomass	Shoot/root
	df	F	F	F	F
AC	1	0.63	2.72	1.83	2.90
LT	1	0.30	0.03	0.07	6.63*
Soil	1	4.30*	18.14***	12.17***	44.31***
Fertilizer	1	42.92***	92.65***	55.68***	139.48***
Plant species	2	199.22***	536.48***	609.26***	6.07**
AC*LT	1	0.01	1.20	1.08	0.00
AC*soil	1	3.14	0.11	0.03	0.03
AC*Fertilizer	1	1.48	0.00	0.06	0.10
AC*plant species	2	2.53	0.10	0.05	1.95
LT*soil	1	0.11	4.02*	3.47*	0.00
LT*Fertilizer	1	3.19	10.57***	11.46***	4.09*
LT*plant species	2	0.90	4.56**	4.45**	0.01
Soil*plant species	2	8.65***	17.34***	20.46***	1.10
Soil*Fertilizer	1	10.70***	4.00*	1.26	42.48***
Fertilizer*plant species	2	31.48***	27.74***	14.01***	0.94
AC*LT*soil	1	0.11	0.08	0.11	0.57
AC*LT*plant species	2	0.59	0.32	0.25	6.07***
AC*LT*fertilizer	1	0.10	0.99	1.00	0.43
LT*soil*fertilizer	1	0.07	0.54	0.39	0.02
AC*soil*fertilizer	1	0.40	1.00	0.62	0.98
plant species*soil*fertilizer	2	1.07	0.78	1.07	1.26
AC*LT*soil*fertilizer	1	0.15	0.56	0.61	0.42
AC*LT*plant species*fertilizer	2	3.12	68.18***	72.46***	5.08*
r^2		0.79	0.90	0.89	0.62

*$p<0.05$; **$p<0.01$; ***$p<0.001$

Table 2 ANOVA table of *F*-values for effects of earthworms (AC and LT), soil and fertilizer on root, shoot and total biomass and shoot-to-root ratio *of Trifolium dubium* (total df=78), *Poa annua* (total df=74) *and Veronica persica* (total df=55)

		T. dubium				P. annua				V. persica			
		Root biomass	Shoot biomass	Total biomass	Shoot/ root ratio	Root biomass	Shoot biomass	Total biomass	Shoot/ root ratio	Root biomass	Shoot biomass	Total biomass	Shoot/ root ratio
	df	F	F	F	F	F	F	F	F	F	F	F	F
LT	1	.	9.10**	10.95**	1.34	.	0.4	0.31	4.66*	.	6.51*	6.20**	2.17
Soil	1	35.71***	117.69***	135.03***	11.56**	5.93*	2.43	1.04	24.05***	5.46*	1.53	3.04	22.86***
Fertilizer	1	29.40***	7.99**	4.79*	29.63***	99.80***	87.66***	48.25***	153.33***	0.26	15.41***	12.03**	36.70***
AC*LT	1	.	.	.	3.18**	.	.	.	0.31	.	.	.	5.68**
LT*soil	1	.	9.54**	10.11**	1.79	.	0.38	0.42	4.91*	.	0.14	0.01	0.03
LT*Fertilizer	1	.	1.64	2.00	2.23	.	13.66**	14.74***	1.81	.	6.22**	6.65**	0.72
Soil*Fertilizer	1	6.26*	9.97**	8.31**	17.82***	4.96*	1.27	0.40	19.94***	4.69	0.03	0.20	16.20***
AC*LT*fertilizer	1	.	3.07*	3.09*	2.19	.	0.02	0.00	.	.	0.07	0.10	0.32
r^2		0.49	0.68	0.73	0.52	0.61	0.63	0.51	0.76	0.15	0.46	0.47	0.71

*$p<0.05$; **$p<0.01$; ***$p<0.001$

effect on shoot biomass and total biomasses varied with plant species (significant LT × plants species interaction, $p<0.01$), soil type (significant LT × soil interaction, $p<0.05$) and fertilizer (significant LT × fertilizer interaction, $p<0.001$). Its effects on the shoot/root ratio also varied with fertilizer addition (significant LT × fertilizer interaction, $p<0.05$). Further the presence of both earthworm species affected the shoot/root ratio and this effect varied with plant species (significant AC × LT × plant species interaction, $p<0.001$). Finally we found that the shoot/root and total biomasses were affected by the soil type and fertilizer but plant species differed in their responses (significant soil × plant species and fertilizer × plant species interactions, $p<0.001$). These general results and the significant interactions involving the plant species justify analysing separately for each plant species the effects of the presence of *L. terrestris*, soil type and fertilization (Table 2).

Shoot biomass

L. terrestris increased the shoot biomass of *T. dubium* (+26%) but decreased the shoot biomass of *V. persica* (−24%) (Table 2). Mineral fertilization increased the shoot biomasses of *T. dubium, V. persica* and *P. annua* (Table 2) by 24%, 46% and 48% respectively. The shoot biomass of *T. dubium* was 140% higher in the nutrient poor soil than in the nutrient rich one. The significant LT × fertilizer interaction for the shoot biomasses of *V. persica* and *P. annua* (Table 2) showed that effect of LT on these plants species varied with mineral fertilization. LT reduced the shoot biomasses of *V. persica* (−37%) and *P. annua* (−10%) when fertilizer was added (Fig. 1). The significant LT × Soil interaction, (Table 1 and LSmeans comparisons) indicated that the effect of LT on shoot biomass of *T. dubium* varied with soil type. It increased the shoot biomass of the legume (+130%) only in the nutrient rich soil.

Root biomass

Mineral fertilization decreased the root biomasses of *P. annua* (−50%) and *T. dubium* (−25%). The root biomasses of the three plant species were affected by soil type with the highest root biomass in the nutrient rich soil for *P. annua* and *V. persica* while *T. dubium*

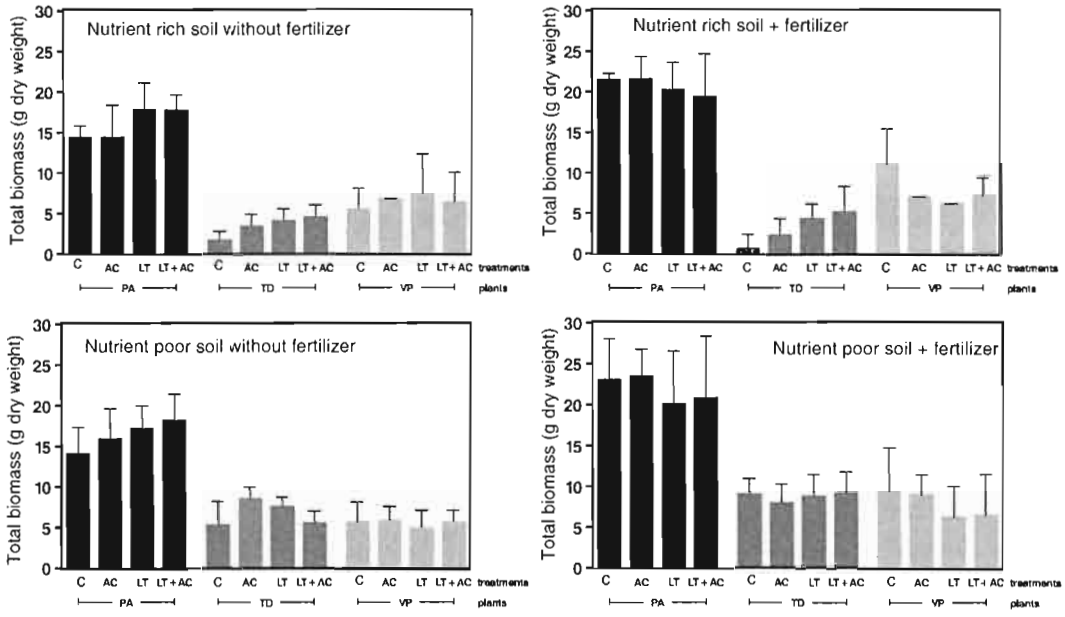

Fig. 1 Effects of earthworms, soil type and fertilizer on the total biomasses of *P. annua* (PA), *T. dubium* (TD) and *V. persica* (VP). Abbreviations: C, control treatment; AC, *A. caliginosa* only; LT, *L. terrestris* only; LT + AC, combined treatment with *A. caliginosa* and *L. terrestris*

showed higher root biomass in the nutrient poor soil (Table 2).

Total biomass

LT increased by 26% the total biomass of *T. dubium* but decreased the total biomass of *V. persica* by 24%. Mineral fertilization increased the total biomass of *T. dubium* (16%), *P. annua* (31%) *and V. persica* (41%) (Table 2). Significant interactions between LT and fertilizer were found for the total biomasses of *P. annua* and *V. persica* suggesting that effects of LT on these plant species varied with mineral fertilization (Table 2). Without mineral fertilization, LT increased the total biomass of *P. annua* (+18%) while no significant effect of this earthworm was found when the fertilizer was added. For *V. persica* LT reduced (−48%) the total biomass when the fertilizer was added but without mineral fertilization it did not show significant effect (significant LSmeans comparisons and Fig. 1). For *T. dubium* the significant LT × Soil interaction (Table 2) showed that the effect of LT on the total biomass of this plant species varied with soil type. In the nutrient rich soil the presence of LT increased significantly the total biomass of *T. dubium* (+121%) while, no significant effect of LT was found in the nutrient poor soil (Fig. 1). Moreover, in the nutrient rich soil without earthworms, the mineral fertilization reduced (−55%) the growth of the legume (Fig. 1). The effect of soil type on the total biomass of *T. dubium* varied with fertilizer addition (Table 2, significant Soil × Fertilizer interaction). In the nutrient poor soil, the addition of fertilizer increased (+22%) the total biomass of the legume while no significant effect of fertilizer was found in the nutrient rich soil (Fig. 1).

Shoo/root ratio

The presence of LT increased by 21% the shoot/root ratio of *P. annua*. Shoot/root ratio of *V. persica* was increased (+32%) when both earthworm species were present (Fig. 2 and LSmeans comparisons). Moreover the shoot/root ratios of the three plant species were significantly affected by the soil type and mineral fertilization. Their shoot/root ratios were higher in the nutrient poor soil than in the nutrient rich one (Fig. 2). Mineral fertilization increased the shoot/root ratio of *T. dubium* (193%), *V. persica* (194%) and *P. annua* (335%). Effects of LT on the shoot/root ratio of *P. annua* varied with the soil type (significant LT × soil interaction, Table 2). LT increased (+27%) the shoot/root ratio of *P. annua* only in the nutrient poor soil (Fig. 2 and LSmeans comparisons).

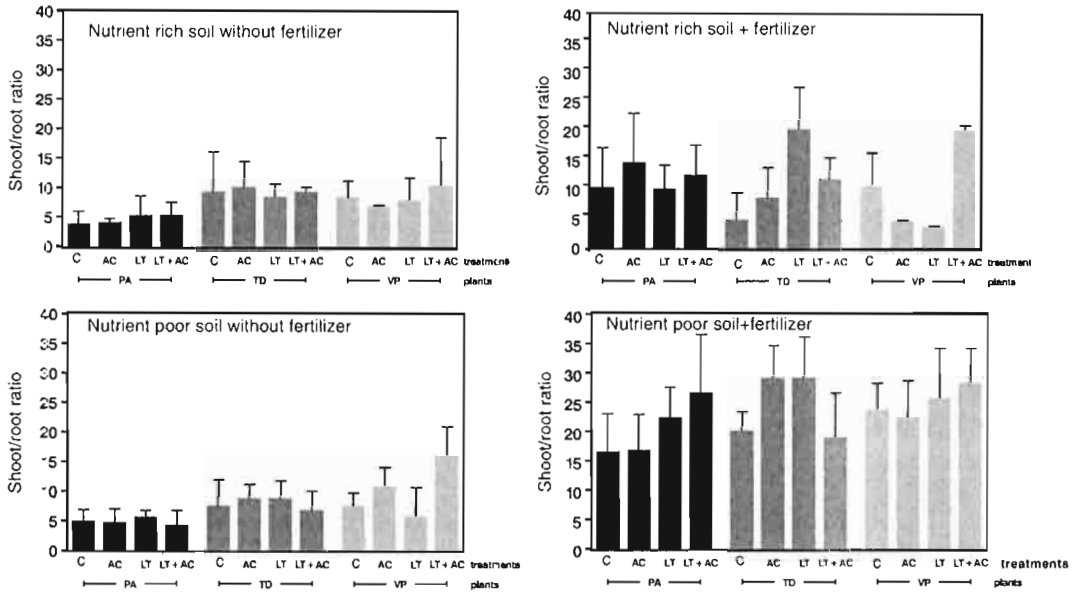

Fig. 2 Effects of earthworms, soil type and fertilizer on the shoot/root ratio of *P. annua* (PA), *T. dubium* (TD) and *V. persica* (VP). Abbreviations: C, control treatment; AC, *A. caliginosa* only; LT, *L. terrestris* only; LT + AC, combined treatment with *A. caliginosa* and *L. terrestris*

Discussion

We hypothesized that: both earthworm species should affect (1) plant growth and (2) plant resource allocation only in the nutrient and organic matter poor soil; (3) significant interactive effects of the two earthworm species on plant growth and resource allocation should only be found in the nutrient and organic matter poor soil; (4) the impact of *A. caliginosa* and *L. terrestris* on plants should decrease with mineral fertilization.

It is generally thought that positive effects of earthworms on plant growth are more likely in nutrient poor soils than in nutrient rich soils (Brown et al. 2004). Although only *L. terrestris* showed significant effects, our results contradict this hypothesis. Our first hypothesis was not confirmed since LT, increased the shoot biomass and the total biomass of *T. dubium* only in the nutrient rich soil. Our second hypothesis was confirmed since there was no case where an earthworm species changed the shoot/root ratio in the rich soil but not in the poor soil. For example, for *P. annua* the presence of LT increased the shoot/root ratio but only in the poor soil as predicted. Few significant interactions between the anecic and the endogeic earthworms were found in this study, and these interactions did not depend on the soil type. Consequently, our third hypothesis was not verified. Similarly, our fourth hypothesis was not supported by our results since there was no case where mineral fertilization decreased or suppressed a positive effect of an earthworm species on the growth of a plant species.

Plant growth

Effects of LT on the growth of *T. dubium* varied with the soil type but contrary to our expectation, this earthworm species did not have any significant effects on this plant in the nutrient poor soil. Indeed, LT increased the shoot and total biomasses of *T. dubium* only in the nutrient rich soil and mineral fertilization did not modify these effects (see Fig. 1). These results suggest that LT effect on *T. dubium* was probably not due to an enhancement of mineralization. This hypothesis is supported by the fact that the fertilizer addition had the opposite effect: it increased the total biomass of this plant only in the poor soil. These results should thus be explained by one of the four other mechanisms through which earthworms influence plant growth (Brown et al. 2004; Scheu 2003). A possible mechanism could be the production of growth regulators in the presence of LT (Blouin et al. 2006). Indeed, as microbial biomass is generally higher in clayey soils than in sandy soils (Hendrix

et al. 1998), the activity of LT could have led to a greater production of phytohormones, through the stimulation of bacteria in the nutrient rich soil than in the nutrient poor soil and this resulted in an increase in the growth of *T. dubium*. Moreover, *T. dubium*, as a legume, is less sensitive to an increase in mineralization (Brown et al. 2004; Jenerette and Wu 2004), which further supports our rationale. An alternative explanation for the positive effect of LT on the legume is that this earthworm species could have increased microbial biomass burying organic matter in the soil which could in turn increase the immobilization of mineral nutrient (Van der Heijden et al. 2007) and allowed *T. dubium* to grow better because it is better adapted to nitrogen poor soils. This hypothesis is supported by the fact that the legume had a lower total biomass in the nutrient rich soil than in the nutrient poor one. Finally, the soil type itself did not affect the growth of *P. annua* and *V. persica* suggesting that these plant species are less sensitive to the soil quality than the legume.

Although LT increased the total biomass of *P. annua* without mineral fertilization, it decreased the total biomasses of *P. annua and V. persica* when mineral fertilizer was added. This suggests that, in our experiment, without mineral fertilization, LT affected these plants mostly through an increase in mineralization. An explanation for the negative effect of LT on the total biomass of *P. annua and V. persica* when mineral fertilizer was added is that this earthworm species could have enhanced the loss of added mineral nutrients through the galleries it produced. This suggests that LT could influence plant growth not only through mineralization of organic matter, at least when the soil contains enough organic matter, but also through its effects on nutrient losses (Barot et al. 2007; Dominguez et al. 2004).

Shoot/root ratio

The effect of earthworms on plant resource allocation is poorly documented and understood (Scheu 2003), but increase of the shoot/root ratio in the presence of earthworms has been documented before (Kreuzer et al. 2004; Scheu et al. 1999). In our study, LT effect on *P. annua* shoot/root ratio varied with the soil type, this earthworm species increased the shoot/root ratio only in the nutrient poor soil. The significant effect of the interaction between LT and the soil type on the shoot/root ratio is probably due to the strategies plants have evolved to optimize their resource allocation to their root system to efficiently take up nutrients. Indeed, when mineral nutrients are poorly available it is efficient for a plant to increase locally its root biomass in nutrient rich patches, while, if mineral nutrient availability is high enough in the whole soil, it is more efficient for a plant to decrease its total root biomass (Wilson 1988). However, plants have evolved different thresholds, first, in the local nutrient availabilities triggering local root proliferation, second, in the general nutrient availabilities triggering a change in the shoot/root ratio (Hutchings 1988). In this way, the observed variations of the responsiveness to earthworms of the shoot/root ratio with the soil type would reflect the scale and the precision at which root systems exploit the soil (Campbell et al. 1991). Moreover, the possible local release of plant growth factors (Muscolo et al. 1999) in earthworm casts and local changes in soil structure due to earthworm activities are also likely to influence local root densities as well as whole shoot/root ratios. This suggests that studying thoroughly changes in the allocation to the root system and in the root system architecture in the presence of earthworms and in different soils would provide useful information on root foraging strategies.

Interactive effect of both earthworm species on plant growth

Few significant effects of the interaction between LT and AC were found in our study; moreover the observed effects did not change with the soil type. This suggests that mechanisms through which the two earthworm species might influence plant growth do not interact. In our experiment this is also probably due to the fact that few significant effects of AC were found. Contrarily to other experiments using similar biomass of AC (Kreuzer et al. 2004; Wurst et al. 2003, 2005), no significant effect of this earthworm species was found on plant growth in our study. AC individuals were probably less active than those of LT that produced a lot of casts on the soil surface (personal observations). Another complementary explanation is that AC might have enhanced mineralization or triggered the release of phytohormones in the soil without affecting significantly plant growth.

In this case, AC might have influenced the physiology of these plants, for example the allocation of N to the root and aerial system, without affecting their biomass. This hypothesis is supported by the results of another experiment where AC affected the N content of plant without affecting their growth (Laossi et al. 2009). Other studies are needed to test for this kind of effect.

Conclusion

We have confirmed that earthworm effects (especially *L. terrestris*) on plants change with soil type (Doube et al. 1997) and nutrient supply. However, our results suggest that earthworm effects do not necessarily decrease with soil content in mineral nutrients and that earthworms do not affect plant via mineralization only. Consequently, other mechanisms are influential and new experiments are required to predict when and where the different mechanisms are influential. This is required to be able to predict how earthworm effects on plant growth vary with soil characteristics such as the availability of mineral nutrients, organic matter content and texture. The issue should be tackled by systematically comparing earthworm effect on plant, everything else being equal, in many different soils representing various combinations of these characteristics.

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