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ROOTS, EARTHWORMS AND MYCORRHIZAE: INDIVIDUAL AND INTERACTIVE EFFECTS ON SOME CHEMICAL, PHYSICAL AND BIOLOGICAL PROPERTIES OF SOILS

THESE

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Roots,earthworms and mycorrhizae : individual and interactive effects of some chemical, physical and biological properties of soils

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Abstract

The belowground communities include a large variety of soil organisms showing highly complex interactions across trophic or non-trophic groups. Soil organisms have been recognized to contribute to a wide range of ecosystem services. They can modify soil physical structure and water regimes, enhancing the amount and efficiency of nutrient acquisition by the vegetation and improving plant health. Among the great diversity of soil biota, plant roots, earthworms, and arbuscular mycorrhizal fungi (AMF) that form a symbiosis with plant roots, are key components. However, only few studies tried to assess the individual or interactive effects of earthworms, AMF and roots, on soil properties and how they influence plant growth. This PhD thesis will therefore principally focus on the contribution of these three soil organisms with respect to their effects on soil nutrient content and on soil structure.

The objectives of this thesis were to assess separately and in combination the effects of endogeic earthworms (*Allolobophora chlorotica*), AMF (*Glomus intraradices*) and leek plants (*Allium porrum*) on some soil physical (soil macroaggregate stability, shrinkage analysis) and chemical (nutrient content, mainly nitrogen, N and phosphorus, P) properties. Moreover, the effect of the three kinds of organisms on soil biological properties, especially the structure of the bacterial communities, was also studied. As earthworms, mycorrhizae and plants have been shown to modify their surrounding soil, the rhizosphere (the soil fraction influenced by the roots), the drilosphere (the soil fraction influenced by earthworms, i.e. casts and burrow-linings), and the remaining bulk soil were also analyzed. In parallel of their effects on soil parameters mentioned above, biological interactions between the three soil organisms were measured as well, with particular attention on the influence of earthworms and AMF on plant performance (link with the aboveground system).

To reach our objectives, three experiments were designed. The first experiment was conducted in a climate chamber and used a compartmental design consisting of microcosms separated vertically into two parts with a nylon mesh to prevent the roots to pass through, but not AMF. The soil used was a loamy Anthrosol that was maintained under phosphorus (P) limited conditions in order to promote the AMF-root symbiosis. We measured soil structure through shrinkage analysis and the percentage of water stable macroaggregates, the total and available phosphorus, total carbon and nitrogen content in the plants and in the different soil fractions as well as bacterial community structures. The second experiment was performed in order to test whether the effects of the three organisms were different according to the P concentration in the soil. The design was similar to the previous experiment; except that it was conducted in a glasshouse and that P fertilization treatment was performed using 5mM KH_2PO_4. We focussed our measurements on the different form of P (total, organic and available P) coupled with enzymatic activity measurements (phosphatase activity at different pH). Finally, the third experiment was set up in order to better characterize the shrinkage results obtained in the first experiment. This last experiment was conducted in a glasshouse but the experimental design was simplified as microcosms were not compartmented. The first 30 cm of

an unstable and sensitive to crusting silt loamy Luvisol was used in order to test if the response of soil organisms observed in the first experiment (see below) is verified. In addition to leek plants, a second plant, the petunia (*Petunia hybrida*), was added in order to test for the effects of different root architectures. Again, we measured soil structure through shrinkage analysis and the percentage of water stable macroaggregates. Moreover, in every experiment, biological interactions between AMF, plant roots and earthworms were assessed by measuring plant and earthworm biomass as well as the mycorrhization rate and the external hyphal length.

The results of this thesis are not presented by experiment but according to the studied soil properties. The effect of plant roots, AMF and earthworms on soil properties are therefore presented in the following order: i) chemical (chapters 2 and 3), ii) physical (chapters 4 and 5), and iii) biological soil properties (chapter 6).

Chemical soil properties were mainly influenced by plant roots and AMF. Available P decreased in the presence of plant roots in the first experiment, but no difference with unplanted pots was measured in the second experiment. In addition, results of the third experiment showed different effect of petunia and leek on available P in the bulk soil. Mite infection (second experiment) or different soils (third experiment) may explain the results. As for plant roots, AMF generally decreased P availability in the soil except in the second experiment. The effect of earthworm on chemical nutrient in the bulk soil was not significant, but drilosphere soil contained higher concentration of P and N.

Physical soil properties were mainly affected by plant roots and earthworms. Plant roots improved soil structure by decreasing soil density and increasing soil stability. Moreover, soil structure was differently affected by the different root architecture of petunia and leek. During the two experiments centred on soil physics, AMF did not significantly influenced soil structure but positively interacted with plant roots. Plants had therefore the greatest positive impact on soil structure, and AMF seem to have a positive synergistic effect by accentuating the effect of plant roots. Contrarily to plant roots, endogeic earthworms negatively affected soil structure, mainly by decreasing soil stability and increasing the bulk soil density (i.e. compaction).

Biological properties, i.e. bacterial community structures, were affected by plant roots, AMF and earthworms, either directly in the bulk soil (AMF and plant roots) or indirectly via the drilosphere soil (earthworms).

Overall, biological interaction between the three soil organisms showed that AMF had no significant effect on earthworm biomass or survival and that earthworms did not influenced the mycorrhization rate or the hyphal length density of AMF. Earthworm survival was besides negatively affected by leek roots. Root exudates are supposed to be responsible of such a result. Finally, the effects of AMF or earthworms on plant performance varied according to the experiments. AMF positively affected shoot and/or root biomass (experiments 1 and 3) or had no effect (experiment 2), whereas earthworms had no significant effect on plant growth. Moreover, the effects of AMF and earthworms on N or P content in the shoots were contrasting, depending probably on limiting soil nutrient content.

Abstract

In conclusion, although we studied the relationships between only three soil organisms, we showed that biotic interactions occurring in the soil are highly complex. Moreover, the three experiments highlighted the importance of measuring physical and chemical soil parameters when studying soil organism interactions and their influence on plant growth. From this point of view, an integrated approach aiming at measuring the effects of biological processes (e.g. interactions between soil living organisms) and physico-chemical ones (e.g. shrinkage analysis) is widely encouraged and seem, according to our results, very useful and promising for future studies.

Keywords

Leek (Allium porrum), petunia (Petunia hybrida), arbuscular mycorrhizal fungi (AMF), Glomus intraradices, endogeic earthworms, Allolobophora chlorotica, drilosphere, rhizosphere, bulk soil, plant biomass, biological interactions, belowground interactions, nutrient availability, phosphorus (P), phosphatase activity, P fertilization, shrinkage analysis, soil porosity, structural stability, soil structure, DGGE, Biolog Ecoplate, N:P ratio

Résumé

Les communautés des êtres vivants dans le sol incluent une grande variété d'organismes qui sont liés entre eux par des interactions complexes au niveau des chaînes trophiques ou non trophiques. Les organismes du sol sont reconnus pour leurs contributions à un grand nombre de services des écosystèmes. Ils peuvent modifier la structure du sol et son régime hydrique, accroître la quantité et l'efficacité d'acquisition des éléments nutritifs par la végétation et ainsi améliorer la santé des plantes. Parmi la grande diversité d'êtres vivants du sol, les vers de terre, les racines des plantes, et les Champignons Arbusculaires Mycorhiziens (CAM) vivant en symbioses avec les racines, sont des éléments clés. Cependant, il n'existe que peu d'études visant à estimer l'effet individuel ou les interactions entre les vers de terre, les CAM et les racines sur les propriétés du sol et leur influence sur la croissance des plantes. Ce travail de thèse va donc principalement s'intéresser à la contribution de ces trois organismes du sol sur la structure du sol et la teneur en éléments nutritifs de celui-ci.

Les objectifs de cette thèse étaient d'évaluer les effets séparés ou conjoints de vers de terre endogés (*Allolobophora chlorotica*), des CAM (*Glomus intraradices*) et des racines du poireau (*Allium porrum*) sur certaines propriétés physiques (stabilité des macroagrégats du sol, analyse de retrait), et chimiques (teneur en éléments nutritifs, principalement l'azote, N et le phosphore, P) du sol. De plus, l'effet de ces trois catégories d'organismes sur des propriétés biologiques du sol a également été étudiée en se focalisant sur la structure des communautés bactériennes présentes dans le sol. Il a été démontré que les vers de terre, les CAM et les plantes modifient le sol qui les entoure. Le sol rhizosphérique (la fraction de sol directement influencée par les racines), le sol drilosphérique (la fraction de sol influencée par les vers de terre, à savoir les turricules, fèces et parois de galeries) ainsi que la fraction de sol restante ont ainsi également été analysées. En parallèle à leurs effets sur les paramètres du sol susmentionnés, les interactions biologiques entre les trois organismes du sol ont aussi été mesurées, avec une attention particulière portée sur l'influence des vers de terre et des CAM sur la croissance des plantes (lien avec le système aérien).

Pour répondre à ces objectifs, trois expériences ont été conçues. La première expérience a été menée en chambre climatisée en utilisant un design expérimental à deux compartiments, à savoir des microcosmes séparés verticalement en deux par une toile de nylon afin d'empêcher les racines de traverser la toile tout en permettant aux CAM de le faire. Le sol utilisé pour cette expérience était un Anthrosol à texture limoneuse qui a été maintenu en condition de carence en phosphore afin de favoriser la symbiose entre le champignon et la plante. Nous avons mesuré la structure du sol au moyen des analyses de retrait et du pourcentage de stabilité de macroagrégats stables à l'eau, ainsi que le phosphore total et disponible pour les plantes, les teneurs en azote et carbone total dans les plantes et les différentes fractions du sol. Les structures de communautés bactériennes ont aussi été mesurées. La deuxième expérience a été réalisée afin de tester si les effets des trois organismes étaient différents en fonction de la teneur en phosphore dans le sol. Le design utilisé était identique à la première expérience, excepté qu'elle a été réalisée en serre et qu'un traitement fertilisation en

phosphore (ajout de 5mM de KH_2PO_4) a été ajouté dans cette expérience. L'accent a été mis sur des mesures de différentes formes de phosphore dans le sol (total, organique et disponible), couplées à des mesures d'activités enzymatiques (activité de la phosphatase à différents pH). Finalement, la troisième expérience a été établie afin de mieux caractériser les résultats des analyses de retrait obtenus lors de la première expérience. Cette dernière expérience a également été conduite sous serre mais au moyen d'un design expérimental simplifié. Les microcosmes n'étaient pas séparés en deux parties. Cette fois, nous avons utilisé les 30 premiers centimètres d'un Luvisol à texture limoneuse fine, instable et sensible à la battance afin de tester si la réponse des organismes du sol, observée lors de la première expérience (voire ci-dessous), se vérifiait. En plus du poireau, une seconde plante, le pétunia (*Petunia hybrida*) a été ajoutée à l'expérience afin de tester l'effet de deux systèmes racinaires différents. A nouveau, nous avons mesuré la structure du sol au moyen des analyses de retrait et du pourcentage de macroagrégats stable à l'eau. Par ailleurs, au cours de chaque expérience, les interactions biologiques entre CAM, vers de terre et plantes ont été évaluées en mesurant les biomasses des plantes et des vers de terres, ainsi que le taux de mycorhization et la longueur des hyphes externes des CAM.

Les résultats de cette thèse ne sont pas présentés par expérience, mais en fonction des propriétés du sol étudiées. L'effet des racines, des CAM et des vers de terre sur les propriétés du sol est donc présenté dans l'ordre suivant : i) propriétés chimiques (chapitres 2 et 3), ii) propriétés physiques (chapitres 3 et 5) et iii) propriétés biologiques (chapitre 6).

Les propriétés chimiques du sol ont été principalement influencées par les racines et les CAM. Le phosphore disponible a diminué en présence des plantes dans la première expérience, mais aucune différence n'a été mesurée dans la deuxième expérience. Par ailleurs, les résultats de la troisième expérience ont montré des effets différents selon l'espèce de plante (poireau et pétunia) sur la disponibilité de P dans le sol. Des attaques d'acariens (deuxième expérience) ou les différences entre les deux sols utilisés (troisième expérience) peuvent expliquer ces résultats. Comme pour les plantes, les CAM ont généralement diminué la disponibilité du P dans le sol, excepté dans la deuxième expérience. L'effet des vers de terre sur les éléments nutritifs du sol n'a pas été significatif, mais le sol drilosphérique contenait une plus grande concentration d'azote et de phosphore disponible.

Les propriétés physiques du sol ont principalement été affectées par les racines et les vers de terre. Les racines ont amélioré la structure du sol en diminuant la densité du sol et en augmentant la stabilité des macroagrégats. De plus, la structure du sol a été influencée différemment en fonction de la présence du poireau ou du pétunia. Durant les deux expériences axées sur la physique du sol, les CAM n'ont pas influencé significativement la structure du sol, mais ils ont positivement interagi avec les racines. Les plantes ont donc eu un impact très fort et positif sur la structure du sol, et les CAM semblent avoir eu un effet synergique positif en accentuant l'effet des racines des plantes. Contrairement aux racines, les vers de terre endogés ont influencé négativement la structure du sol. Ils ont principalement diminué la stabilité et augmenté la densité du sol (i.e. compaction).

Les propriétés biologiques, i.e. la structure des communautés bactériennes, ont été influencées par les racines, les CAM et les vers de terre, soit directement dans le sol distant (CAM et racines) soit indirectement via le sol drilosphérique (vers de terre).

Résumé

De manière générale, les interactions biologiques entre les trois organismes du sol ont montré que les CAM n'avaient pas d'effets significatifs sur la biomasse ou la survie des vers de terre et que les vers de terre n'ont également pas influencé le taux de mycorhization ou la longueur des hyphes externes des champignons. La survie des vers de terre a été en outre négativement influencée par les racines du poireau. Les exsudats racinaires du poireau semblent être responsables de ces résultats. Finalement, les effets des vers de terre ou des CAM sur les plantes varient selon les expériences. Les CAM ont positivement influencé les biomasses des racines et les parties aériennes (tiges et feuilles) dans les expériences 1 et 3 ou n'ont eu aucun effet significatif (expérience 2), tandis que les vers de terre n'ont eu aucun effet significatif sur la croissance des plantes. De plus, les effets des CAM et des vers de terre sur les teneurs en N et P dans les parties aériennes des plantes sont contrastés et dépendent probablement de la teneur en éléments nutritifs limitants dans les sols.

En conclusion, bien que nous ayons étudié la relation entre trois organismes uniquement, nous avons montré que les interactions biotiques qui se déroulent dans le sol sont très complexes. De plus, les trois expériences ont mis en évidence l'importance de mesurer des paramètres physiques et chimiques du sol lorsque l'on étudie les interactions entre les organismes du sol et leur influence sur la croissance des plantes. Dans ce sens, une approche intégrée, visant à mesurer les effets des processus biologiques (interactions des organismes vivants du sol) et physico-chimiques du sol (par exemple analyses de retrait), est largement encouragée et semble, d'après nos résultats, très utile et prometteuse pour de futures études.

Mots-clés

Poireau (Allium porrum), petunia (Petunia hybrida), champignons arbusculaires mycorhiziens (CAM), Glomus intraradices, vers de terre endogés, Allolobophora chlorotica, drilosphère, rhizosphère, sol distant, biomasse végétale, interactions biologiques, interactions souterraines, disponibilité des éléments nutritifs, phosphore (P), activité de la phosphatase, fertilisation en phosphore, analyse de retrait, porosité du sol, stabilité structurale, structure du sol, DGGE, Biolog Ecoplate, N:P ratio

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General introduction

Roxane Milleret

"In many ways the ground beneath our feet is as alien as a distant planet. The processes occurring in the top few centimetres of Earth's surface are the basis of all life on dry land, but the opacity of soil has severely limited our understanding of how it functions" Sugden et al. (2004) in Soils - The Final Frontier, special issue of *Science***.**

The present manuscript is composed of a general introduction, followed by five chapters and a general conclusion. The general introduction describes the broad context and the key ecological questions of the research conducted during the thesis. Because the next chapters are written in a paper form, specific topics of each of them will be described more precisely within specific introductions.

The present introduction is separated into several parts. In the first part, an overview of the soil and its living constituents is given with a focus on the importance of soil fauna in the belowground system and how soil biota may affect some soil properties. The general concepts of the soil properties developed during the thesis are thereafter explained. In the next section, the direct effects of the three studied organisms on these soil properties are described. By acting together in the soil, these soil organisms are in close relationships and interact with each other. These interactions are summarized in the third section. Finally, the next three sections, describe the lack of knowledge in this field of research, give the objectives of the thesis and how chapters are organised.

The soil and its living constituents

At the interface between the atmosphere, the lithosphere, the hydrosphere and the biosphere, the soil, or more precisely the pedosphere, forms a thin mantle over the Earth's surface. The pedosphere is commonly defined as a multiphase system, composed of different elements as mineral material, living or dead organic matter (organisms and litter), water and gases. Under the influence of several factors (climate, parent material, topography, organisms and time), these biotic and abiotic elements interact through numerous physicochemical and biological processes and lead to many soil properties as soil texture and structure, water content, organic matter, ionic equilibrium, etc (Coleman et al. 2004; Gobat et al. 2004). Until recently, the soil has been considered by soil biologists as a black box composed of a

multitude of decomposers. However, with the development of new molecular tools and the acknowledgement that the soil was a huge reservoir of biodiversity (Andre et al. 1994), the key role of soil organisms in soil functioning has been better recognized.

The soil contains a wide amount of living organisms, ranging from eye-invisible microbes (bacteria and fungi) to macro fauna (termites, earthworms, etc.) with in between organisms of intermediary size as microfauna (protozoa, nematodes, etc.) and mesofauna (microarthropods, enchytraeids, etc.). Furthermore, although plants are primary producers and determine the amounts of carbon that enter the system via the aboveground system (Wardle 2002), the root system, as an heterotrophic part of the plant, may also be considered as a soil organism, as they are in close relationship with other soil biota. All these soil organisms are interacting together in very complex trophic and non trophic webs (Bardgett 2005; Coleman 2008; Wardle 2002) that affect in turn the aboveground system (Coleman 2008; Van der Putten et al. 2001; Wardle 2002). Moreover, they utilize the soil as a habitat and a source of energy (Bardgett 2005) and have therefore a strong effect on soil properties.

Soil organisms have been recognized to contribute to a wide range of ecosystem services (Barrios 2007; Costanza et al. 1997; Food and Agriculture Organisation (FAO) 2001). First, they act as the primary driving agents of nutrient cycling, regulating the dynamics of soil organic matter, soil carbon sequestration and greenhouse gas emission. Second, they modify soil physical structure and water regimes, enhancing the amount and efficiency of nutrient acquisition by the vegetation and improving plant health. Among all these services, we will principally focus on the contribution of soil organisms with respect to their effects on i) soil nutrient content and ii) on soil structure.

i. Soil nutrients are chemical compounds that are essential to the growth and survival of primary producers. Among them, nitrogen (Nasholm et al. 2009), phosphorus (Hinsinger 2001) and potassium (Maser et al. 2002) are of major importance as they are usually limiting for plant growth. However, other soil macro- or micronutrients (e.g. calcium, magnesium, sulfur, manganese, iron, zinc, etc.) are also essential (Marschner 1995). Soil nutrients can be supplied from different sources as the decomposition of organic matter, the exchange of ions in the soil solution, the mineral weathering, processes of adsorption/desorption of ions from soil particles, and the biological N fixation or atmospheric deposition. Moreover, most soil nutrients can be found in different chemical forms in the soil. Consequently, soil nutrients are generally not homogeneously distributed within the soil, varied among soil types and are not necessary directly in the available form for primary producers. Plant roots have therefore to adapt and to find strategies (e.g. the extension of the root network, the secretion of organic acids, the synthesis of phosphatase enzymes, the symbiosis with mycorrhizal fungi) to acquire nutrients in available forms.

ii. According to Kay et al. (1988) in Amezketa (1999), the soil structure is defined in terms of forms and stability. The structural form refers to the more or less heterogeneous arrangement of solid and void space existing in the soil at a given time. The structural stability refers to the ability of the soil to retain its arrangement when exposed to stress conditions. Soil structure is generally

associated with the concept of soil aggregation, i.e. the process by which aggregates of different sizes are linked and held together by different organic and inorganic materials. Several models of aggregation exist (see Amezketa 1999 or Six et al. 2004 for reviews). These models suppose that clay particles are attached to organic molecules by polyvalent cations, in order to form the primary particles. Primary particles are further bound together into microaggregates (< 250 μm) which are in turn bound into macroaggregates (> 250 μm). Several factors are needed to bind particles together. Tisdall and Oades (1982) distinguished three types of binding agents. The persistent binding agents (i.e. the humified organic matter and/or the polyvalent metal cation complexes) are implied in the formation of microaggregates. Macroaggregation depends mainly of temporary (i.e. fungal hyphae and roots) and transient (i.e. microbial- or plant- derived polysaccharides) binding agents. Moreover, aggregation and structural stability are influenced by internal factors (e.g. clay mineralogy, organic matter) and external factors as time, climate or biological parameters. Among biological parameters, Jastrow and Miller (1991) distinguished between the effect of soil organisms themselves, their activities and their by-products (root exudates, extracellular or enzymes secretions, etc.).

Among the great diversity of soil biota, earthworms, arbuscular mycorrhizal fungi (AMF) and plant roots, as ecosystem engineers (Jones et al. 1994), are considered as agents involved in the formation of biological aggregate in temperate soils by Six et al. (2002). They are also responsible for the mobilization, release and cycling of soil nutrients. This thesis will focus on the effects of earthworms, mycorrhizae and plant roots on some soil physical (soil structure) and chemical (soil nutrient content) properties. Next section describes major individual effects of these three soil organisms on these soil properties.

Direct individual effects of plant roots, AMF and earthworms on soil properties

Direct effects of plant roots

Within the soil, plant roots greatly influence soil properties, mainly in the zone of the soil immediately adjacent to the roots called the rhizosphere (Hiltner 1904). Root-related processes affecting soil structure (reviewed by Angers and Caron 1998) or soil nutrient content can be grouped into several categories. First, by penetrating into the soil, roots affect soil porosity by exerting a radial pressure and by compressing the soil in their vicinity, leading to the realignment of the clay matrix. Root penetration is dependent of the type of root architecture (e.g. degree of branching, thickness of roots, etc.) as demonstrated by Carter et al. (1994). Second, root water uptake affects the soil water regime as wet-dry cycling of adjacent soil is increased. It is therefore generally assumed that the drying of soil by the roots increase the soil stability. Third, the release of organic material by plant roots – the rhizodeposition (review by Nguyen 2003) – acts as binding agents (mainly polysaccharides) and may influence soil nutrient content (through the action of organic acids for instance) and soil structure directly or indirectly (via microbial stimulation which in turn produces metabolic products acting as binding agents, thus stabilizing aggregates). Fourth, as a source of

organic matter, dead plant residues affect also soil structure via decomposition processes. During decomposition processes, the organic matter is fragmented via mineralization, assimilation by microorganisms or humification and is thereafter integrated into aggregates. Finally, soil structure is also influenced by direct root entanglement of soil particles, as demonstrated in many studies (Six et al. 2004; Tisdall and Oades 1982).

Direct effects of AMF

Arbuscular mycorrhizal fungi (AMF) are obligate mutualists that form a symbiosis with up to 80 % of vascular plants such as grasses, herbs, agricultural crops and some legumes. They have been separated from other fungal groups and have been recognized to belong to a new monophyletic phylum named Glomeromycota and more particularly to the class Glomeromycetes (Schussler et al. 2001). There are over 150 described AMF species whose most part belong to the *Glomus* genera (Redecker 2008). These fungi are characterized by structures called arbuscules that grow and ramify treelike within the root cells. These arbuscules are considered to be the site of exchange between the fungus that provides nutrients (mainly nitrogen, N, and phosphorus, P) and the plant that provides carbon (Marschner and Dell 1994; Smith and Read 1997). In addition, AMF have external structures called hyphae that extend into the surrounding soil. The soil directly associated with external hyphae network is called the hyphosphere (Marschner 1995). It is generally assumed that AMF are an extension of the root network system that enhances the efficiency of root nutrient acquisition (Smith and Read 1997).

It has been demonstrated by Rillig and Mummey (2006) that the fungal hyphae influence soil aggregation via three processes. First, AMF affect soil aggregation formation or stabilization physically by the enmeshment of primary particles, organic matter and small aggregates to macro aggregates and the alignment of primary particles (Andrade et al. 1998; Thomas et al. 1993). Second, AMF affect soil aggregation chemically by the secretion of extracellular compounds as mucilages, polysaccharides or glomalin-related soil proteins (Wright and Upadhyaya 1996). These compounds have been shown to act as a glue-substance (Bronick and Lal 2005; Six et al. 2004). Finally, AMF affect soil aggregation biologically by interacting with the soil food web, thus influencing bacterial communities that would in turn influence soil aggregation through the secretion of polysaccharides (Andrade et al. 1998; Andrade et al. 1997; Artursson et al. 2005).

Direct effects of earthworms

Since the work of Darwin (1881), a large amount of literature has described the importance of earthworm activities in the soil functioning system (Edwards and Bohlen 1996; Lavelle and Spain 2001). Earthworms belong to the phylum Annelida in the class of Oligocheta, which consist of 36 families worldwide, but comprise only one third of exclusively terrestrial species. There are over 3500 known earthworm species (Bohlen 2002). They are often grouped into three functional categories based on their morphology, behaviour, feeding ecology, and their microhabitat within the soil (Bouché 1977). First, epigeic species are polyhumic (they prefer organically enriched substrate) and live and feed at the soil surface or within the litter. Second, endogeic species usually inhabit

the mineral soil, feed on soil organic matter and create subhorizontal burrows within the soil. Finally, anecic species exploit both the surface litter as a source of food and the mineral soil. They form vertical burrows and egest their faeces (casts) onto the soil surface. They are the main actors in the formation of the organo-mineral complex.

Earthworms as soil ecosystem engineers (Jones et al. 1994; Lavelle et al. 1997) play a very important role on soil structure and its nutrient content. By creating soil biogenic structures (casts, burrows), which are called drilosphere soil (Lavelle et al. 1997), earthworms influence physical, chemical and biological soil properties. Through casting and burrowing activities, they affect the soil physically, by modifying soil porosity, aggregation, stability, aeration, and hydraulic conductivity (Edwards and Bohlen 1996; Shipitalo and Protz 1989). Through selectively feeding on microorganisms, they also influence soil biological properties by ingesting soil particles and organic matter that are modified through the passage in their gut (Brown et al., 2000). The resulting casts or burrows have been shown to host bacterial communities with a particular structure and composition (Amador and Gorres 2007; Brown et al. 2004; Savin et al. 2004). Finally, earthworms have an impact on soil chemical properties. By feeding selectively in soil regions rich in organic compounds, earthworms, and their gut-associated bacteria enhance organic matter mineralization, which in turn increases the nutrient availability in cast and burrows (Brown et al. 2004; Le Bayon and Binet 2006). They also change the soil chemically by creating the burrow walls that are often lined with clay and external mucus deposited by earthworms (Edwards and Bohlen 1996). These burrow walls can form a stable structure that may persist in soil for several months, or even years (Lee 1985).

Interactions between earthworms, AMF and plant roots

Interactions among earthworms, AMF and plants are not obvious but complex (Fig. 1.1). These interactions can be direct (soil organisms have direct effects between each other) or indirect (soil organisms have their own individual direct effects on soil properties, as discussed in the previous section, that in turn affect the other soil organisms).

Direct interactions between soil organisms result mainly from trophic interactions (Bardgett 2005; Coleman et al. 2004; Wardle 2002). On the one hand, the close relationship between AMF and plant roots has been widely studied (Allen 1992; Smith and Read 1997). It has been demonstrated that AMF, considered as an extension of the root network, lead to increased plant uptake of inorganic N (Hawkins et al. 2000) and available P (Jakobsen et al. 1992). In turn, plants transfer between 10% and 20% of their net photosynthates (i.e. chemical products of photosynthesis) to the fungus (Jakobsen and Rosendahl 1990). AMF infection have also been shown to modify root parameters as root architecture, length, biomass, etc. (Berta et al. 2002; Gamalero et al. 2004; Klironomos 2003; Piotrowski et al. 2004). On the other hand, direct interactions between earthworms and AMF and/or plant roots are less clear. Several authors (reviewed in Brown et al. 2004) highlight a root abrasion and an ingestion of living plant parts by earthworms during bioturbation as well as interaction with plant seeds (ingestion, digestion, burial, dispersal, changes in germination rate). Some authors (El Harti et al. 2001; Krishnamoorthy and

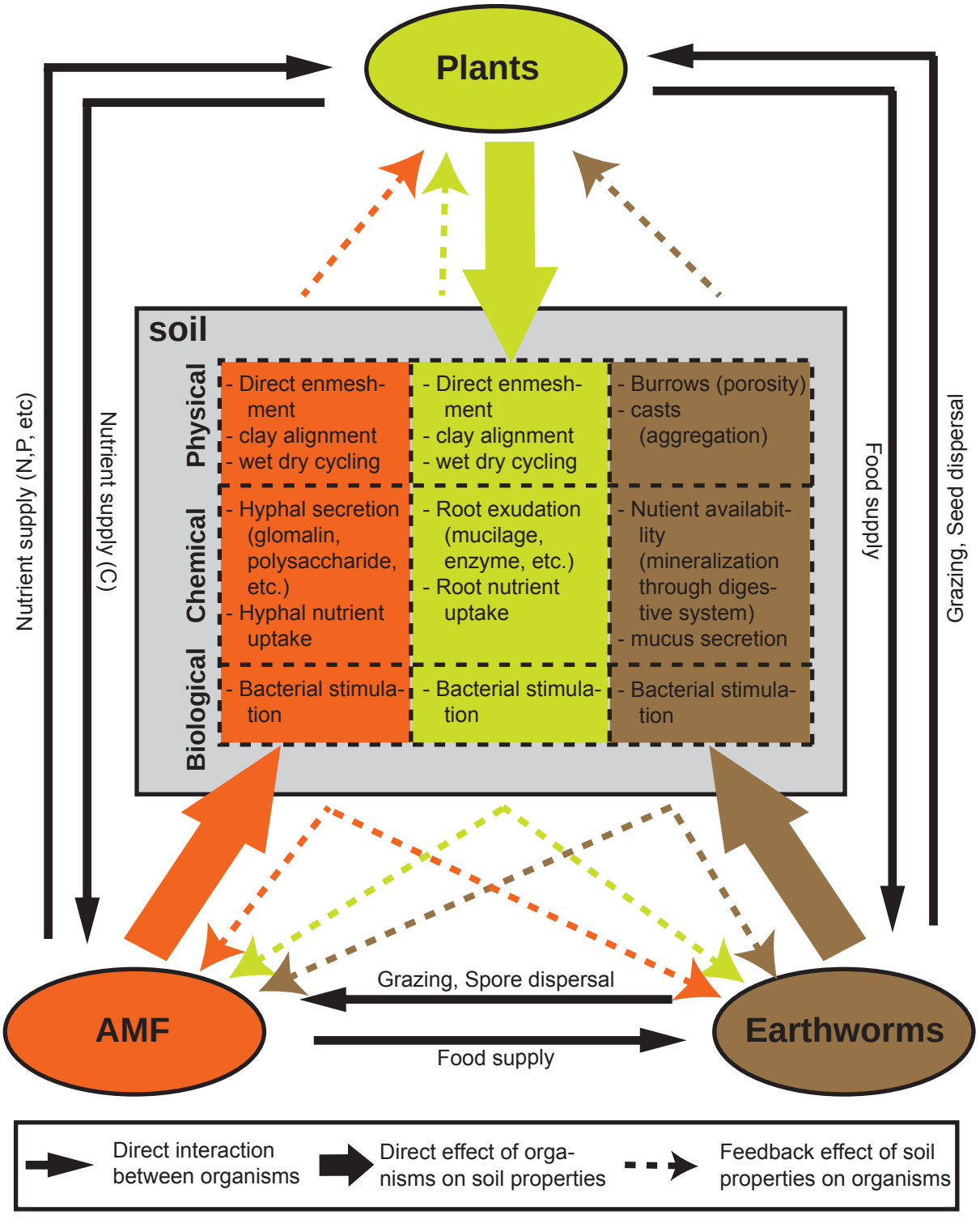

Fig. 1.1 Main relationships between roots, AMF, and earthworms. Relations can be direct (thin continuous arrows) and/or indirect through direct effects of soil organisms on soil properties (large continuous arrows), that in turn affect other soil organisms (thin discontinuous arrows). The grey box represents soil properties as affected by the direct effects of roots, AMF and earthworms as described in section *Direct individual effects of plant roots, AMF and earthworms on soil properties*.

Vajranabhaiah 1986) also suggest the ability of earthworms to excrete plant growth promoting/ regulating substances (e.g. hormones, vitamins, auxins, cytokinins, gibberellins, etc.). However, it is not clear whether these substances are directly excreted by earthworms or by bacteria living within their digestive tract. Earthworms are also known to interact with AMF either by grazing on hyphae or as a vector of spore dispersion either during soil ingestion or when attached to earthworm's cuticle (Bonkowski et al. 2000; Gange 1993; Gormsen et al. 2004; Ortiz-Ceballos et al. 2007; Reddell and Spain 1991).

Through multiple direct individual effects on soil properties or the fraction of the soil they transform (drilosphere, rhizosphere, hyphosphere, remaining bulk soil), soil organisms have indirect interactions one with another. For example, by increasing mineralization in casts and burrow-linings, earthworms may directly enhance the nutrient availability in soil, which in turn indirectly influences the nutrient uptake by roots or hyphae. This effect may depend on the accessibility of these available nutrients for plant or AMF, i.e. the ability of roots to find the earthworm-formed patches of food (casts, faeces, and burrow-linings). In addition, by creating casts and burrows, earthworms graze on AM fungi and the hyphal network may be reduced. This may indirectly affect plant performance as the mycorrhization rate can be reduced which may be unbeneficial for plants grown in P-deficient conditions. These two examples highlight the complexity of studying several organisms, especially in the soil where the action of an organism may give rise to a cascade of processes that will affect other organisms. Moreover, spatiotemporal delays between direct action of one organism in a soil property and the response of a second organism are supposed to exist, which increased our difficulties to have good understandings of the interactions between soil organisms.

Lack of knowledge

As recently mentioned by Eisenhauer et al. (2009) and Wurst et al. (2008), the interacting effects of functionally dissimilar soil organisms on the ecosystem functioning are of particular importance since individual effects of soil organism groups may cancel out each other in combination. Most studies focus on a restricted number of soil actors. Among them, a lot of publications describe the interactions between roots, AMF and their effects on soil physical properties as soil stability or porosity (Hallett et al. 2009; Rillig and Mummey 2006), nutrient availability (Hawkins et al. 2000; Jakobsen 1995; Jansa et al. 2005; Smith et al. 2003) or biological properties as bacterial communities in the rhizosphere (Andrade et al. 1997; Artursson et al. 2005). In parallel, interactions between earthworms and roots on soil properties and plant growth have also been reported (Brown et al. 2004; Fraser et al. 2002; Springett and Gray 1994). Generally, most studies on earthworms looked at the difference between the drilosphere and the bulk soil (Edwards and Bohlen 1996; Shipitalo and Protz 1989; Tiunov and Scheu 1999).

However, only few studies tried to assess the interactive effects of earthworms, AMF and roots, on different soil parameters and plant growth. Table 1.1 summarizes papers studying the single or interactive effects of earthworm and AMF on plant biomass and/or plant N and P content. From this table, several points can be highlighted. First, only six studies have been published since 2002 (I only took into account studies that tried to assess the individual and

Table 1.1 Synthesis of the existing publications that aim at assessing the individual and interactive effects of AMF and earthworms on plant productivity and/or some nutrient content in the vegetative parts. ↑, positive effect (response variable increased); ↓, negative effect (response variable decreased); ns, not significant.

Plant	AMF	Earthworm	Response variable	AMF	Earthworm	Interaction	Reference
Trifolium resupinatum	*G. mossae*	*Pheretima sp.*	Shoot biomass	↑	↑	No	Zarea et al. 2009
			Shoot N amount	↑	↑	No	
Trifolium alexandrinum	*G. mossae*	*Pheretima sp.*	Shoot biomass	↑	↑	No	
			Shoot N amount	↑	↑	No	
Lolium perenne	*G. intraradices*	*Apporectodea caliginosa* + *Lumbricus terrestris*	Shoot biomass	↓	ns	No	Eisenhauer et al. 2009
			Root biomass	↓	↑	No	
			Shoot P amount	ns	ns	No	
			Shoot N amount	ns	ns	No	
Trifolium repens	*G. intraradices*	*Apporectodea caliginosa* + *Lumbricus terrestris*	Shoot	↑	ns	No	
			Root	↑	ns	No	
			Shoot P amount	↑	ns	No	
			Shoot N amount	ns	ns	No	
Plantago lanceolata	*G. intraradices*	*Apporectodea caliginosa* + *Lumbricus terrestris*	Shoot	ns	ns	No	
			Root	ns	ns	No	
Leucaena leucocephala	*G. intraradices* + *G. mossae*	*Pheretima guillelmi*	Total	↑	↑	Yes	Ma et al. 2006
			Shoot P concentration	↑	↑	Yes	
			Shoot N concentration	↑	↑	Yes	
Lolium multiflorum	*G. intraradices* + *G. mossae*	*Pheretima sp.*	Shoot	↓	↑	No	Yu et al. 2005
			Root	ns	ns	No	
			Cd shoot concentration	↑	ns	No	
			Cd root concentration	↑	↑	Yes	
Plantago lanceolata	*G. intraradices*	*Apporectodea caliginosa*	Shoot	ns	↑	No	Wurst et al. 2004
			Root	↓	ns	No	
			Shoot P amount	↑	ns	No	
			Shoot N amount	ns	ns	No	
			Root P amount	↑	ns	No	
			Root N amount	↓	ns	No	
Allium porrum	mixed AMF spores	*Apporectodea caliginosa*	Shoot	ns	↑	No	Tuffen et al. 2002
			Root	ns	↑	No	
			^{32}P shoot amount	↑	↑	No	
			^{32}P root amount	ns	↑	No	

combined effect of the three organisms in a single experiment). Second, no clear evidence of a unique positive, neutral or negative effect of the organisms has been observed. In studies where the effect of earthworm was significant, their presence was generally positive. They increased plant biomass or the amount of N or P in the shoots. The effects of AMF were more contrasted. Positive, negative or no significant effect have been described in the different publications. Interestingly, only two studies found significant interactions between AMF and earthworms. These differences between the studies can be attributed to the diversity of soil types and organism species used in the experiments or the length of each experiment. Finally, all these studies focussed on plant production (biomass measurements) or shoot and root nutrient uptake mainly N and P. The interacting effects of major soil biota on soil properties are consequently largely understudied. From these observations, we conclude that a better knowledge of the role of AMF, earthworms and plant roots and/or their interactions on soil properties is essential to have a clearer understanding of the factors affecting interactions between soil organisms and how they influence plant growth.

Objectives

The aims of this thesis were to assess separately and in combination the effects of endogeic earthworms, AMF and plant roots on some soil physical (soil macroaggregate stability, shrinkage analysis) and chemical (nutrient content, mainly N and P) properties. Moreover, the effect of the three kinds of organisms on soil biological properties, especially the structure of the bacterial communities, was also tested. As earthworms, mycorrhizae and plants have been shown to modifiy their surrounding soil, the rhizosphere, the drilosphere, and the remaining bulk soil were also integrated and analysed. In parallel of their effects on soil parameters, biological interactions between the three soil organisms were also measured, with particular attention on the influence of earthworms and AMF on plant performance (link with the aboveground system).

The general hypotheses are:

- Earthworms, AMF and roots influence soil physical, chemical and bacterial properties,
- The effects of these soil organisms on soil properties are different depending on whether they act individually or in interaction with the others,
- The physical, chemical and biological variables measured in the drilosphere, rhizosphere and the bulk soil are different,
- Biological interactions between these soil organisms occur and affect plant productivity.

Organization of the thesis

The research performed in this thesis is composed of three experiments performed chronically as new questions and ideas arose during the trials (Table 1.2). The first experiment was conducted in a climate chamber and used a compartmental design consisting of microcosms separated vertically into two parts with a nylon mesh to prevent the roots to pass through, but not AMF. The soil used was a loamy Anthrosol (soil nomenclature after IUSS 2006) that was maintained under phosphorus (P) limited conditions in order to promote the AMF-root symbiosis (see Appendix 1 for the experimental design of the first experiment). This experiment aimed to assess the effect

Table 1.2 Organization of the thesis. Experimental setup, soil type, location, duration as well as the factors and measurements of the three experiments and their location in the next chapters.

Exp	Experimental setup	Soil type	Location	Duration	Factors	Measured soil properties	Chapter
1 (Appendix 1)	Microcosm with 2 compartments	Loamy Anthrosol	Climate chamber (University of Neuchâtel)	5, 15 and 35 weeks	- Endogeic earthworm (*Allolobophora chlorotica*) - AMF (*Glomus intraradices*) - Leek (*Allium porrum*) - Time of harvest (5, 15, 35 weeks)	Chemical (available P, total C and N)	2
						Physical ($WSA_{1\text{-}2\ mm}$)	2 and 4
						Physical (shrinkage analysis)	4
						Biological (bacterial community structures by using DGGE and Biolog™ techniques)	6
2 (Appendix 2)	Microcosm with 2 compartments	Loamy Anthrosol	Glasshouse (Botanical garden, Neuchâtel)	45 weeks	- Endogeic earthworm (*Allolobophora chlorotica*) - AMF (*Glomus intraradices*) - Leek (*Allium porrum*) - P fertilization (+/-)	Chemical (Total, organic, inorganic and available P) Acid and alkaline phosphatase activity	3
3 (Appendix 3)	Microcosm	Silt loamy Luvisol	Glasshouse (HEPIA, Lullier)	22 weeks	- Endogeic earthworm (*Allolobophora chlorotica*) - AMF (*Glomus intraradices*) - Leek (*Allium porrum*) - Petunia (*Petunia hybrida*)	Chemical (available P) Physical ($WSA_{1\text{-}2\ mm}$) Physical (shrinkage analysis)	5

of endogeic earthworms (*Allolobophora chlorotica*), mycorrhizae (*Glomus intraradices*) and plants (*Allium porrum*) on several soil physical (structural stability, shrinkage analysis), chemical (nutrient content, C, N, P) and biological (bacterial community structures) properties, and how these effects vary with time.

In order to test if the effects of the three organisms were different according to the P concentration in the soil, the second experiment was performed. This experiment was similar to the previous one; except that it was conducted in a glasshouse in order to have more natural conditions (natural light, seasonal variations, etc.). In this case, P fertilization was performed. This second experiment tried to assess how the three kinds of organisms affect the different forms of P (total, organic and available P) in the soil and how plant production was influenced. Enzymatic activity measurements (phosphatase activity at different pH) were also taken into account (see Appendix 2 for the experimental design).

Finally, the third experiment was set up in order to better characterize the shrinkage results obtained in the first experiment. This last experiment was conducted in a glasshouse but the experimental design was simplified as microcosms were not compartmented (see Appendix 3). The first 30 cm of a silt loamy Luvisol was used in order to test if the response of soil organisms varied with the soil type. In addition, a second plant, the petunia (*Petunia hybrida*), was added in order to test for the effects of different root networks (petunia versus leek).

For a better understanding and clarity of the manuscript, the following chapters are not presented chronologically by experiments but thematically according to the main soil properties analysed (see also Table 1.2):

- Chapters 2 and 3 investigate the impact of earthworms, AMF and plant roots on soil chemical properties as performed in experiment 1 and 2.
- Chapters 4 and 5 focus on their effects on soil physical parameters as analysed in experiment 1 and 3.
- Chapter 6 explores their effects on biological properties as assessed in the first experiment.
- Finally, chapter 7 integrates and summarizes all results of the preceding chapters to get an overview of the individual and interactive effects of soil organisms on soil properties. The general hypotheses are discussed and research perspectives are then proposed.

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2

Root, mycorrhiza and earthworm interactions: their effects on soil structuring processes, plant and soil nutrient concentration and plant biomass

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Abstract

Earthworms, arbuscular mycorrhiza fungi (AMF) and roots are important components of the belowground part of terrestrial ecosystem. However, their interacting effects on soil properties and plant growth are still poorly understood. A compartmental experimental design was used in a climate chamber in order to investigate, without phosphorus (P) addition, the single and combined effects of earthworms (*Allolobophora chlorotica*), AMF (*Glomus intraradices*) and roots *(Allium porrum*) on soil structure, nutrient concentration and plant growth. In our experimental conditions, plant roots improved soil structure stability (at the level of macroaggregates) whereas earthworms decreased it. AMF had no effect on soil structure stability but increased P transfer from the soil to the plant and significantly increased plant biomass. Earthworms had no direct influence on P uptake or plant biomass, and the N:P ratio measured in the shoots indicated that P was limiting. Interactions between AMF and earthworms were also observed on total C and N content in the soil and on total root biomass. Their effects varied temporally and between the different soil compartments (bulk soil, rhizosphere and drilosphere). After comparison with other similar studies, we suggest that effects of earthworms and AMF on plant production may depend on the limiting factors in the soil, mainly N or P. Our experiment highlights the importance of measuring physical and chemical soil parameters when studying soil organism interactions and their influence on plant performance.

Keywords

arbuscular mycorrhizal fungi (AMF); endogeic earthworms; macroaggregate stability; rhizosphere; drilosphere; plant biomass; nutrient availability; N:P ratio

Introduction

Belowground biotic interactions are known to influence soil fertility and plant growth by changing soil nutrient cycling and the physical environment (Wardle 2002). Belowground communities include a large variety of organisms showing highly complex interactions across trophic or non-trophic groups (Coleman 2008). Among the great diversity of soil biota, earthworms, arbuscular mycorrhizal fungi (AMF) and plant roots are key components (Six et al. 2002). However, their interacting effects on soil properties are still poorly understood.

Root networks enhance soil porosity as well as soil aggregation through direct entanglement of particles and/or secretion of mucilages that help adhere particles together (Six et al. 2004; Tisdall and Oades 1982). As a result of these root-induced changes in soil structure, plant growth may be affected (Angers and Caron 1998).

AMF-plant symbiosis is based on the reciprocal transfer of plant-derived carbon to the fungus and soil-derived nutrients from the fungus to the plant (Smith and Read 1997). For plants, this symbiosis is particularly important in soils with a low nutrient content (Marschner and Dell 1994; Smith et al. 2004). In particular, it has been demonstrated in pot experiments that association with AMF, considered as an extension of the root network, leads to increased plant uptake of inorganic nitrogen (Hawkins et al. 2000) and available phosphorus (P_a) (Jakobsen et al. 1992). Moreover, AMF influence soil aggregation and, consequently, soil structure stability by binding and enmeshing soil particles into larger aggregates (see Rillig & Mummey (2006) for a review).

Earthworms are also major components of the soil system. Their activities influence soil properties and plant production through numerous ways (Brown et al. 2004). For example, earthworms may disperse AMF spores by soil ingestion or by transporting them attached to their cuticles. Moreover, when burrowing, earthworms may affect the development of the mycelium by grazing and therefore by disrupting the contact of the external hyphae from the roots. Direct grazing of AMF may either be deleterious by reducing the fungal biomass or advantageous by stimulating fungal growth due to an enhanced organic matter mineralization caused by fauna (Ortiz-Ceballos et al. 2007). In parallel, earthworms influence plant growth physically by changing the structure of the soil. Burrowing and casting activities are known to affect soil porosity and aggregate size distribution, stability, aeration and hydraulic conductivity (Edwards and Bohlen 1996; Shipitalo and Protz 1989). Finally, earthworms influence plant growth by changing the spatiotemporal availability of nutrients - mainly phosphorus (Le Bayon and Binet 2006), nitrogen (Devleeschauwer and Lal 1981) and carbon (Guggenberger et al. 1996) - in their casts and burrow walls.

Very few studies have focused on the combined effects of plants, AMF and earthworms, and most of these were devoted to plant biomass measurement. In two different studies (Tuffen et al. 2002; Wurst et al. 2004), earthworms enhanced plant growth while mycorrhizae either reduced root biomass or had no effect. Therefore, very little information is available concerning the effects of plant roots, AMF and earthworms as well as their interactions on soil chemical and/or physical parameters that influence soil fertility and plant growth.

The main aims of this study were to assess separately and in combination the effects of earthworms (*Allolobophora chlorotica,* Savigny), AMF (*Glomus intraradices,* Schenk & Smith) and plant roots (*Allium porrum,* L.) on soil structure and available nutrient concentration in the bulk soil, the rhizosphere soil (the part of the soil influenced by roots) and the drilosphere soil (the part of the soil influenced by earthworm secretions and castings). The interacting effects of earthworms and AMF on plant growth were in turn investigated. Finally, the influence of time on these interactions was tested with three different experiment durations. This study was conducted in a climate chamber and without phosphorus addition in order to promote the symbiosis between AMF and plants.

The choice of the different components of our study was motivated by several reasons. Regarding the leek plant, previous studies showed a positive response to AMF inoculation in agricultural soils and in pot experiment (Sorensen et al. 2005, 2008) and it has been a model plant for different soil fauna interaction studies with mycorrhiza (Tuffen et al. 2002; Warnock et al. 1982). The fungus *Glomus intraradices* is widely used for laboratory studies and commonly found in the soil environment. Finally, *Allolobophora chlorotica* was selected due to its behaviour as an endogeic species (i.e. they feed on soil organic matter, live mainly near plant roots and burrow horizontally and vertically within the soil) and thus its interaction with the root and fungal network.

Our working hypotheses were that earthworms, AMF and plant roots would show individual and interacting effects. We suppose that AMF would enhance P uptake by the plant roots and that earthworms would improve soil structure and porosity. By co-occurring in the soil media, we suppose that these organisms would show synergistic effects and improve soil fertility that in turn would influence the aboveground system by increasing plant production. The effects of these organisms were thought to vary temporally and to be dependent of soil compartments.

Materials and methods

Experimental setup

Before the experiment, the organo-mineral horizon of an Anthrosol (ISSS 1998) was collected at the botanical garden of Neuchâtel (Switzerland). This is a loamy soil (45.3% sand, 28.0% silt and 26.7% clay), containing 20.7% carbonates and showing a pH_{KCl} of 7.8. The soil contained 521 µg g^{-1} total phosphorus (P), of which 32.2 µg g^{-1} were in available forms (mainly $H_2PO_4^-$ and HPO_4^{2-}). The soil was air-dried, sieved (2 mm) and stored at 20 °C.

A compartmental microcosm design was set up. It consisted of a PVC tube (35 cm height and 15 cm internal diameter) separated into two equal parts by a nylon mesh (25 µm, ©SEFAR, Switzerland). Each side of the microcosm was filled with six successive 5 cm thick layers of soil remoistened at 22% water content. In order to eliminate AMF from the soil, microcosms were first sterilized with γ-irradiation (between 42 kGy and 82 kGy; Studer Hard, Dänikon, Switzerland) and stored at 4°C (McNamara et al. 2003). A 20 ml soil suspension (100 g of soil dispersed in 1000 ml of autoclaved distilled H_2O and filtered on 11 µm paper) was then added to re-inoculate the sterilized soil with microorganisms, but without AMF (Koide and Li 1989).

We defined eight treatments representing all possible combinations of the presence/absence

of the three following factors: (1) three leek plantlets (L): *Allium porrum* var. Mercure, 18 days old, sown in sterilized conditions, (2) AMF inoculum (A): 30 g of culture sand substrate mixed with *Glomus intraradices* spores and hyphae (the treatments without AMF received 30 g of a sterilized inoculum, autoclaved at 121°C over 1 h and gamma-irradiated) and (3) 5 endogeic earthworms (E): *Allolobophora chlorotica* of equal size and total biomass of 1.3 g (± 0.1 g). Earthworms were previously hand-collected in the botanical garden of Neuchâtel (Switzerland) using the hot mustard extraction technique (Lawrence and Bowers 2002), and were relieved of their gut contents before their introduction into the microcosm. The nylon mesh, separating the microcosm into two parts, retains the roots but allows hyphae to pass through. Therefore our compartmental design permitted to separate the individual effect of AMF from the root effect.

The three factors were allocated to the microcosms in two steps. First, leek plants were attributed randomly on one side of each microcosm. Therefore, each microcosm contained both levels of the plant factor (absence/presence). Then, the four possible combinations of mycorrhiza and earthworm factors (A, E, A+E and Control) were randomly allocated to the microcosms. For the microcosms receiving the AMF treatment, 30 g of inoculum was added before introducing the leek plantlets in one side of each microcosm. However, the AMF could colonize both sides by passing through the nylon mesh. For the microcosms receiving the earthworm treatment, groups of five earthworms of equal biomass were prepared and added to both sides of the microcosms. This corresponds to a high density of 650 individuals m^{-2} or 150 g m^{-2} which is respectively around 2.3 times or 1.5 times higher than in a maize crop according to Le Bayon and Binet (1999). A fourth factor, time of harvest (t), was considered in order to take time variation into account. Complete destruction of the microcosm was performed after 5, 15 or 35 weeks. These three time points combined with the eight treatments gave 24 treatments utilizing 12 microcosms (two treatments per microcosm, see above). Each treatment was replicated six times resulting in a total of 72 microcosms.

All microcosms were kept in a climate chamber (Normoflex, KR 11C/200S10, Schaller Uto AG, Bern, Switzerland) under the following conditions: photoperiod 16/8 h (day/night), temperature 18 ± 2 °C and 50% humidity. Microcosms, randomly redisplayed in the climate chamber every week, were watered twice a week using a modified Hoagland's nutrient solution without P in order to promote the AMF-plant symbiosis. Every three weeks, each microcosm was adjusted to equal soil water content with deionised water by weighing.

Harvesting and measurements

After 5, 15 or 35 weeks, leek shoots were cut at ground level, pooled, weighed and air-dried. Three different soil compartments were removed from the microcosms: (1) Rhizosphere Soil (RS), still adhering to the roots after gentle shaking, was collected by rubbing roots carefully on a 2 mm mesh sieve; (2) Drilosphere Soil (DS) was obtained by sampling faeces and the few millimetres-thick layer around the earthworm burrows; (3) the remaining Bulk Soil (BS) was thoroughly mixed. Soil samples were air-dried before analyses were performed. For BS, 10 g of fresh soil were frozen for the measurement of soil water stability and hyphal length density (see below). After rhizosphere soil collection, roots were carefully washed, mixed, weighed

and stored at 4°C in a lactoglycerol-mix made-up of lactic acid : glycerol : deionised water (1:1:1). Earthworms were hand-collected, counted and weighed.

Mycorrhizae analysis

To measure AMF root infection, roots were first cleared in 10% KOH, acidified in 1% HCl and stained in 0.05% Trypan blue in lactoglycerol. The AMF colonisation was determined on three root samples at 250x magnification using a modified line intersect method (McGonigle et al. 1990). Moreover, hyphal length density (HLD) was determined by using an aqueous extraction and a membrane filter technique modified after Jakobsen et al. (1992). Briefly, three replicates of a 4 g soil sample were first dispersed in a sodiumhexametaphosphate solution (35 g l^{-1}) and shaken for 30 s (end-over-end). After 30 minutes, the suspension was decanted quantitatively through a 40 µm sieve to retain hyphae, roots and organic matter, transferred with 200 ml of deionised water into a 250 ml flask and shaken vigorously by hand for 5 s. After 1 min, 4 x 1 ml aliquots (10 sec interval) were taken and pipetted onto Millipore RAWG02500 membranes (Millipore, Bedford MA, USA). The filter was finally stained in 0.05% Trypan Blue. HLD was estimated with a gridline intersect method at 250 × magnification (Newman 1966).

Physical analysis

The water-stable soil macroaggregates in the 1-2 mm size class ($WSA_{1\text{-}2mm}$) were determined using the wet-sieving apparatus (Kemper and Rosenau 1986). A 250 µm sieve was filled with a 4 g sample of 1-2 mm air-dried aggregates. The samples were then moistened by capillarity with deionised water for 10 minutes and wet-sieved 10 minutes more with a stroke length of 19 min^{-1}. The WSA corresponded to the amount of macroaggregates (> 250 µm) remaining on the sieve and was expressed as a percentage of the total initial mass of soil after correction for the weight of coarse particles (> 0.25 mm).

Chemical analysis

After Kjeldahl oxidation, total P concentration was determined colorimetrically at 880 nm using the molybdate procedure (Murphy and Riley 1962) on 2 g of pulverised shoots. Soil samples were measured for available phosphorus forms according to Olsen et al. (1954). Available P (P_a) was extracted from subsamples of 2 g of soil with sodium bicarbonate $NaHCO_3$ (0.5 N, pH 8.5) and determined at 880 nm using the Murphy and Riley method (see above).

Total nitrogen (N) and carbon (C) were measured using a CHN-analyser (CHN EA1108-Elemental analyser, Carlo Erba Instruments) on 2 mg of pulverised shoots or on 10 mg of the three soil compartments - BS, RS and DS.

Statistical analysis

All the statistical analyses were performed with R 2.6.0 (R Development Core Team 2007). For variables with only one measurement per microcosm (shoot and root weights and AMF root colonization), two or three-way ANOVAs were performed with earthworms (E), time of harvest (t) and/or AMF (A) as factors. Tukey HSD tests were performed for multiple comparisons between treatments. When both sides of the microcosm or soil compartments were concerned, partly nested ANOVAs were performed in order to take into account the fact that many samples were in the same microcosm. In this case, leek or soil compartments were

considered to be nested within the microcosm. Consequently, the ANOVA model contained earthworms, AMF and time of harvest as between-microcosms factors and leek or soil compartments as within-microcosms factors.

Results

Soil biota responses

Throughout the entire experiment, no AMF colonization of roots was found in non-AMF-treated samples. The interaction earthworm (E) x time (t) had a significant effect on the percentage of root colonization by AMF (A) ($F_{1,36}$ = 3.54, P = 0.04). After 5 weeks without earthworm, 69.7 % (SE = 6.4 %) of roots were colonized by AMF, whereas only 54.0 % (SE = 3.7 %) of roots were colonized when earthworms were present in the microcosm. At the end of the experiment this difference decreased to 61.5 % (SE = 2.5 %) and 58.3 % (SE = 2.9 %) root colonisation without and with earthworms, respectively.

The hyphal length density (HLD) differed among treatments. The HLD was significantly higher in the side of the microcosm containing Leek roots (L) than in the side with the hyphal network separated from the leek roots (mean HLD with L: 2.0 m g^{-1} soil (SE = 0.1 m g^{-1} soil), mean HLD without L: 1.8 m g^{-1} soil (SE = 0.1 m g^{-1} soil); $F_{1,72}$ = 5.02, P = 0.03).

There was a significant time effect on the total number of earthworms ($F_{2,72}$ = 14.99, P < 0.001). The mean individual number of earthworms per microcosm side was 3.9 (SE = 0.3) after 5 weeks, 5.5 (SE = 0.6) after 15 weeks and 14.0 (SE = 2.6) after 35 weeks. The mean weight of all earthworms present in each side of the microcosms after 5 weeks was 1.2 g (SE = 0.1 g) in each side of microcosm. This mean weight significantly increased with time ($F_{2,72}$ = 3.69, P = 0.04). After 35 weeks, the mean weight of the earthworms reached 1.7 g (SE = 0.2 g). In addition, the presence of the leek negatively affected earthworm biomass (mean E biomass with L: 1.1 ± 0.2 g; mean E biomass without L: 1.7 ± 0.3 g; $F_{1,72}$ = 15.9, P < 0.001). The interaction L x t also showed a significant effect on the earthworm mean weight ($F_{2,72}$ = 3.80, P = 0.03).

Physical analysis

Earthworms, leeks and time showed a highly significant effect on the percentage of water-stable macroaggregates in the 1-2 mm size class ($WSA_{1\text{-}2mm}$) (Table 2.1). With earthworms, the percentage of $WSA_{1\text{-}2mm}$ was significantly lower (25.8 ± 1.0%) than without earthworms (31.6 ± 1.1 %) (Fig. 2.1). On the contrary, this percentage was significantly higher with leek roots (31.3 ± 1.2 %) than without leek (26.2 ± 0.9 %). The interactions E x t, L x t and L x A were also significant. AMF and leek together enhanced the percentage of water stable macroaggregates compared to leek alone (Fig. 2.2a).

Chemical analyses

AMF, leek and time significantly affected the amount of available P (P_a) in the bulk soil (Table 2.1). Available P in the bulk soil was significantly lower when AMF and leek were added (Fig. 2.3a). The L x A interaction was also significant. Available P was lower when both AMF and leek were present in the microcosm (Fig. 2.2b). Moreover, the interactions A x t and L x t showed a significant effect on P_a in the BS samples. No significant main effects were observed for the total N content in the bulk soil

Table 2.1 Partly nested ANOVA showing the effect of earthworms, AMF, leek and time on the percentage of water stable macroaggregates (WSA_{1-2mm}), available P (µg g^{-1}) and total carbon and nitrogen content (mg g^{-1}) in the bulk soil. E: earthworms; A: AMF; L: leek plants; t: time of harvest; df: degrees of freedom; MS: mean square; ***: $P< 0.001$, **: $P< 0.01$*: $P<0.05$, ns: not significant.

Bulk soil analysis	*df*	Physical parameters		Chemical parameters					
		WSA_{1-2mm}		Available P		Carbon content		Nitrogen content	
		F	*P*	F	*P*	F	*P*	F	*P*
Between microcosms									
E	1	25.30	***	1.83	ns	0.74	ns	2.04	ns
A	1	1.70	ns	81.71	***	1.25	ns	1.51	ns
t	2	16.72	***	4.37	*	9.45	***	0.59	ns
E x A	1	2.69	ns	0.63	ns	9.1	**	8.72	**
E x t	2	11.88	***	1.61	ns	11.18	***	1.63	ns
A x t	2	0.47	ns	10.08	***	5.55	**	1.47	ns
Residuals (MS)	62	47.75		6.76		0.08		$1.14 \cdot 10^{-3}$	
Within microcosms									
L	1	28.49	***	40.78	***	1.65	ns	0.76	ns
E x L	1	1.07	ns	0.85	ns	1.08	ns	0.04	ns
A x L	1	4.64	*	6.69	*	1.25	ns	1.34	ns
L x t	2	30.91	***	24.44	***	1.56	ns	1.18	ns
Residuals (MS)	67	32.70		6.84		0.06		$0.30 \cdot 10^{-3}$	

(Fig. 2.3b). However, the E x A interaction was significant. Without earthworms, the total N amount in BS was lower with AMF compared with the non-AMF treatment, whereas with earthworms, total N content was higher with AMF (Fig. 2.2c). Total C showed similar pattern than total N (Figs 2.2d and 2.3c). Moreover, total C in the BS was significantly affected by time and the interactions E x t and A x t (Table 2.1). Contrary to P_a, the presence of leek had no effect on the amounts of C and N in the bulk soil.

Because of a lack of sufficient RS and DS soil material after 5 weeks, analyses on the differences between soil compartments were only made on data sets collected at 15 and 35 weeks. Available P, total N and total C were significantly different between the soil compartments (Table 2.2). Drilosphere soil contained more P_a and total N and less total

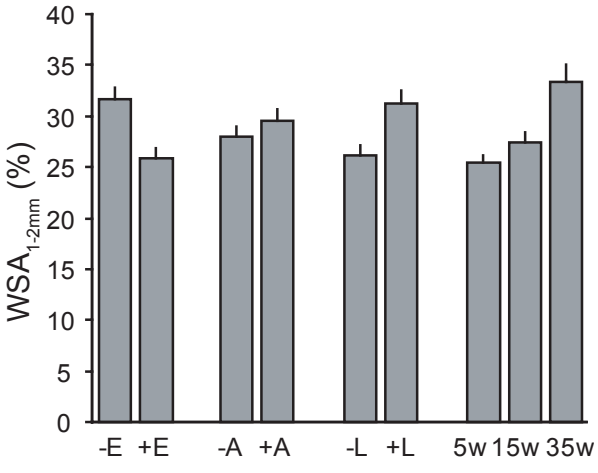

Fig. 2.1 Main effects of the presence of earthworms (E, *Allolobophora chlorotica*), AMF (A, *Glomus intraradices*), leek (L, *Allium porrum*) and time of harvest with complete destruction of microcosms after 5, 15 and 35 weeks (5w, 15w, 35w) on the percentage of water-stable macroaggregates in the 1-2 mm size class (WSA_{1-2mm}). Bar represents mean ± SE.

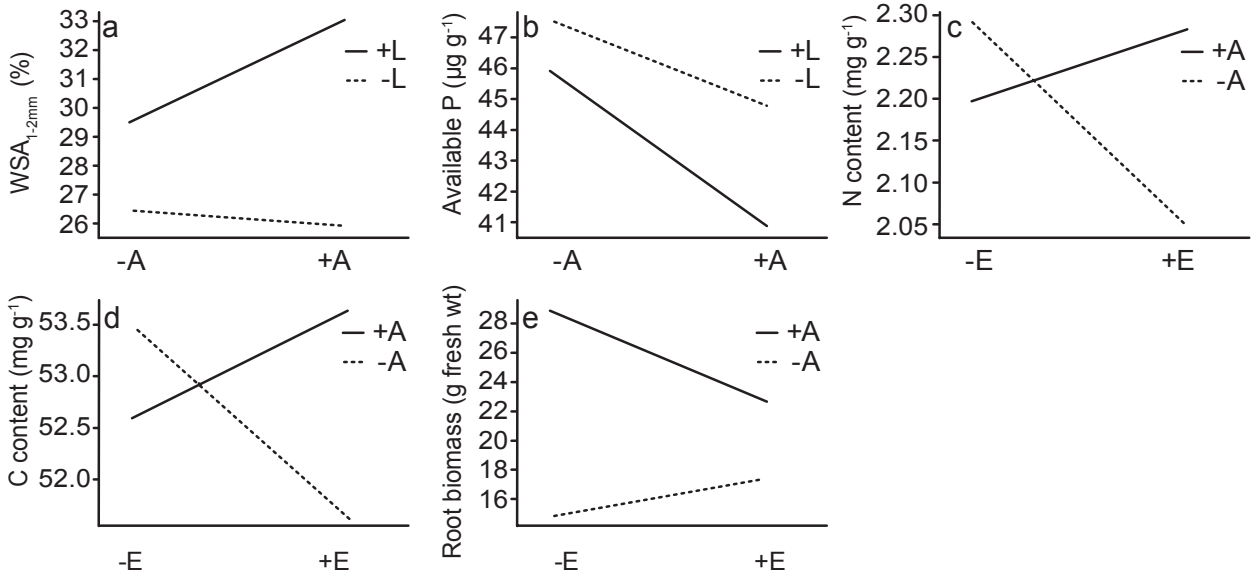

Fig. 2.2 Plot of the significant interactions of AMF (A, *Glomus intraradices*) and leek (L, *Allium porrum*) on (a) the percentage of water stable macroaggregates in the 1-2 mm size class ($WSA_{1\text{-}2mm}$) and (b) available P in the bulk soil and the significant interactions between AMF (A, *Glomus intraradices*) and earthworms (E, *Allolobophora chlorotica*) on (c) N content (mg g^{-1}) in the bulk soil, (d) C content (mg g^{-1}) in the bulk soil and (e) root biomass (g fresh wt).

C compared to rhizosphere and bulk soil (Fig. 2.4). As in the bulk soil analysis, time, AMF and their interaction had a significant effect on P_a. Available P was lower with AMF (39.8 ± 1.0 µg g^{-1}) compared with non-AMF treatment (47.3 ± 0.6 µg g^{-1}) (Fig. 2.4a). No effect of AMF was observed on total C and N (Fig. 2.4b and 2.4c).

Table 2.2 Partly nested ANOVA showing the effects of AMF, time and soil compartment on available P (µg g^{-1}), and carbon and nitrogen content (mg g^{-1}). A: AMF; t: time of harvest; sc: soil compartment (bulk soil, rhizosphere or drilosphere soil); df: degrees of freedom; MS: mean square; ***: $P< 0.001$, **: $P< 0.01$*: $P<0.05$, ns: not significant.

	df	Available P		Carbon content		Nitrogen content	
		F	*P*	F	*P*	F	*P*
Between microcosms							
A	1	56.87	***	0.38	ns	0.01	ns
t	1	28.92	***	17.00	***	1.50	ns
A x t	1	7.35	*	0.92	ns	0.17	ns
Residuals (MS)	20	17.62		0.02		$0.53 \cdot 10^{-3}$	
Within microcosms							
sc	2	10.38	***	16.11	***	4.17	*
sc x A	2	0.59	ns	0.41	ns	1.87	ns
sc x t	2	6.58	**	1.70	ns	0.72	ns
Residuals (MS)	42	9.26		0.05		$0.56 \cdot 10^{-3}$	

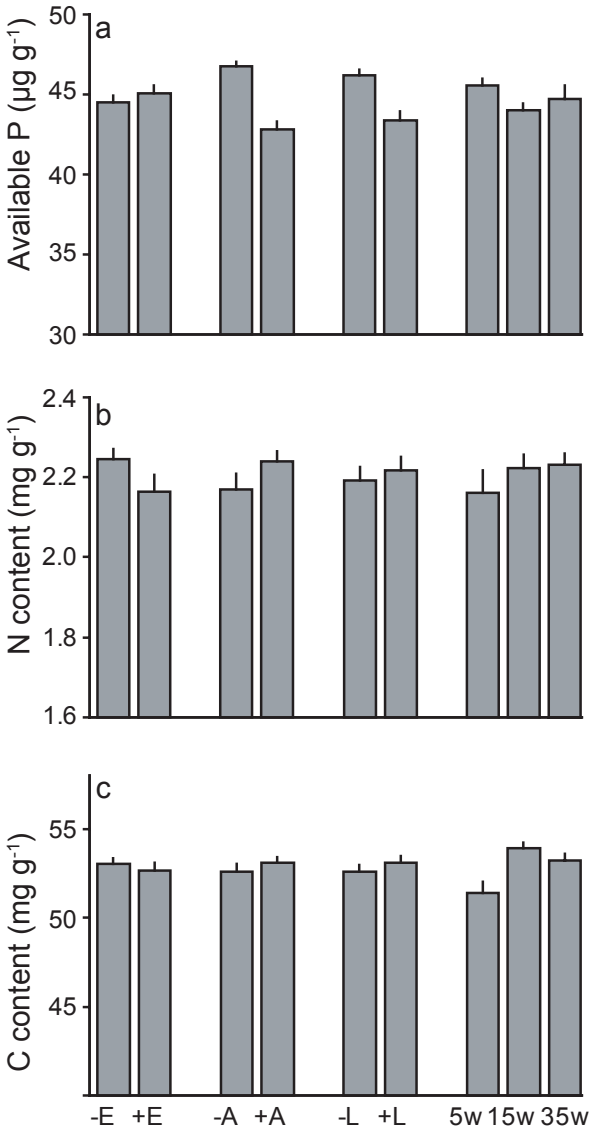

Fig. 2.3 Main effects of earthworms (E, *Allolobophora chlorotica*), AMF (A, *Glomus intraradices*), leek (L, *Allium porrum*) and time of harvest with complete destruction of microcosms after 5, 15 and 35 weeks (5w, 15w, 35w) on (a) available P (µg g^{-1}), (b) total N content (mg g^{-1}) in the bulk soil and (c) total C content (mg g^{-1}) in the bulk soil. Bar represents mean ± SE.

Earthworm and AMF contribution to leek biomass and leek nitrogen and phosphorus content

Total fresh root biomass varied significantly with AMF and time (Table 2.3). The interactions A x t and E x A also significantly affected root biomass. The presence of earthworms had a positive effect on root weight without AMF, whereas with AMF this effect was negative (Fig. 2.2e). After 35 weeks, the fresh root weight was greater with AMF treatment, intermediate with earthworm treatment and minimal with leek alone. AMF, time and their interaction had a significant effect on total fresh shoot weight. After 35 weeks and in the presence of AMF, fresh shoots were more than three times heavier than without mycorrhizal symbiosis. In the shoots, AMF treatment had a significant positive effect on total P and N concentration. The total P and N concentration decreased significantly with time. AMF and time had a significant effect on the N:P ratio (Table 2.3). This ratio was approximately two times lower when leeks were inoculated with AMF, both after 15 and 35 weeks.

Discussion

Root, earthworm and mycorrhiza contribution to the soil structuring processes

In the present study, plant roots and earthworms showed significant but different effects on water-stable aggregation. The macroaggregates (i.e. aggregates > 250 µm measured in the 1-2 mm size class) were more stable with plants, indicating a beneficial root effect that was likely due to root exudates (mucilages) acting like cement on particles or direct root enmeshment of fine particles into stable macroaggregates (Tisdall and Oades 1982). In contrast, macroaggregates were less stable with earthworms. Previous studies demonstrated that fresh casts are less stable than the surrounding soil, but become more stable after aging and drying (Shipitalo and Protz 1989). In our long-term experiment (35 weeks), earthworms occupied almost all the

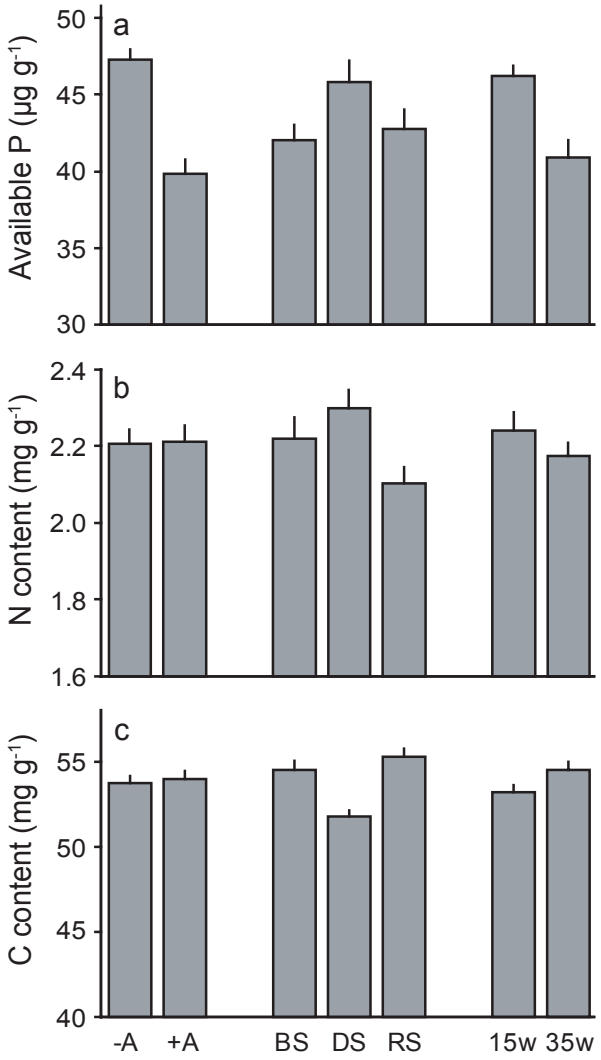

Fig. 2.4 Main effects of AMF (A, *Glomus intraradices*), soil compartment (BS: Bulk Soil, DS: Drilosphere Soil, RS: Rhizosphere Soil), and time of harvest with complete destruction of microcosms after 15 and 35 weeks (15w, 35w) on (a) available P (µg g^{-1}), (b) total nitrogen content (mg g^{-1}) and (c) total carbon content (mg g^{-1}). DS and RS samples after 5 weeks were not available. Bar represents mean ± SE.

soil column volume. They may have rearranged the soil particles and their fresh faeces, combined with frequent watering, caused the soil to be less stable compared to microcosms without earthworm. Earthworms may also have changed the aggregate size distribution by either diminishing soil macroaggregates and/or increasing soil microaggregates during particle ingestion. For tropical earthworms, Blanchart et al. (2004) described compacting and decompacting species. In complex and highly diverse systems such as the soil, this may reflect the importance of studying many ecological categories of earthworms interacting with each other and other soil biota. Future researches that focus on microaggregate stability (Six et al. 2004) or shrinkage analysis (Boivin et al. 2006) would be useful to better understand the system and the role of earthworms, as well as all other soil fauna, on soil structure.

Compared to plant and earthworm treatments, AMF treatment showed no significant effect on water-stable macroaggregates. This is in contradiction with previous studies that showed improved soil stability with AM fungi colonization due, for example, to glomalin-related soil protein (GRSP) (Rillig et al. 2002). As previously explained with earthworms, AMF may have modified the aggregate size distribution of soil and enhanced soil microaggregates that were not measured here (Rillig, pers. comm.). However, working with similar compartmental systems, Andrade et al. (1998) showed analogous results: the percentage of water-stable aggregates was higher in the AMF + plant treatment, lower in the control and intermediate in the AMF or plant single treatments. Moreover, we demonstrated a significant interaction between AMF and plant. In interaction with leek roots, the external hyphae improve the percentage of water-stable macroaggregates, which demonstrate a beneficial effect of the mycorrhizal-plant association. Thus, we suggest that the combination of root exudates, glomalin secretion from AMF and the enmeshing role of both roots and fungi greater improved soil stability instead of AMF alone. This is in accordance with previous studies of Piotrowski et al. (2004) and Schreiner et al. (1997) who showed that the effect of AMF on soil aggregation depends on the interaction between plant and fungal species.

Table 2.3 Mean values (± SE) of total root biomass (g fresh wt), total shoot biomass (g fresh wt), total P (mg g^{-1}), total nitrogen (N) (mg g^{-1}) and N:P ratio in shoots after each time of harvest (5, 15 or 35 weeks). Total P, total N and N:P ratio data after 5 weeks not available. Different letters of superscript mean a significant difference at $P<0.05$ in the same column (Tukey HSD). E: earthworm; A: AMF; t: time of harvest.

Time of harvest	E	M	Root	Shoot			
			Root biomass	Shoot biomass	Total P	Total N	N:P ratio
5 weeks	+	-	0.42 (0.14)^{ef}	0.82 (0.14)^{d}	na	na	na
	-	+	0.43 (0.26)^{ef}	2.12 (1.14)^{d}	na	na	na
	+	+	0.40 (0.07)^{ef}	2.04 (0.27)^{d}	na	na	na
	-	-	0.21 (0.06)^{f}	0.67 (0.14)^{d}	na	na	na
15 weeks	+	-	5.12 (1.12)^{def}	8.83 (1.19)^{d}	1.39 (0.11)^{c}	35.27 (2.31)^{a}	25.76 (1.56)^{a}
	-	+	13.37 (0.79)^{de}	47.12 (2.67)^{c}	2.92 (0.08)^{a}	36.87 (1.46)^{a}	12.63 (0.27)^{bc}
	+	+	14.01 (1.06)^{d}	53.79 (1.67)^{c}	3.09 (0.06)^{a}	38.27 (0.87)^{a}	12.45 (0.44)^{bc}
	-	-	4.89 (1.13)^{def}	8.80 (1.13)^{d}	1.50 (0.24)^{bc}	33.93 (2.18)^{a}	24.49 (2.90)^{a}
35 weeks	+	-	46.67 (4.93)^{bc}	59.92 (4.82)^{c}	0.74 (0.04)^{d}	15.69 (2.29)^{bc}	21.02 (2.36)^{a}
	-	+	72.88 (4.06)^{a}	232.33 (11.85)^{a}	2.04 (0.12)^{b}	20.39 (0.93)^{bc}	10.04 (0.23)^{c}
	+	+	53.58 (5.55)^{b}	207.44 (11.85)^{b}	2.01 (0.23)^{b}	22.28 (2.59)^{b}	11.16 (0.69)^{c}
	-	-	39.23 (3.58)^{c}	61.99 (4.37)^{c}	0.62 (0.03)^{d}	12.01 (1.41)^{c}	19.07 (1.61)^{ab}
ANOVA *P*-value							
E			0.30	0.27	0.70	0.12	0.35
A			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
t			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.01**
E x A			**0.01**	0.37	0.75	0.74	0.61
E x t			0.25	0.06	0.93	0.59	0.65
A x t			**<0.001**	**<0.001**	0.27	0.09	0.16

Mycorrhiza, plant root and earthworm contributions to nutrient availability and consequences on leek biomass

Overall, we observed a significant positive effect of AMF and leek roots on nutrient availability, which improved plant biomass. When grown with AMF, plant shoots and roots were heavier and soil P_a levels were lower. Despite studies showing different responses of plant growth with AMF inoculations (Smith et al. 2004; van der Heijden et al. 2006), our experiment confirmed that under P limitation, AMF enhanced significantly plant growth through nutrient acquisition, particularly through the available P in the soil (Marschner and Dell 1994).

In contrast to AMF and contrarily to our expectations, earthworms showed no main significant effect on nutrient availability and plant biomass. These results contradict previous studies that aimed at determining mycorrhizae-earthworm interactions (Tuffen et al. 2002; Wurst et al. 2004). In particular, Wurst et al. (2004) pointed out a negative effect of mycorrhizae on root biomass of *Plantago lanceolata* after a 10-week experiment but no earthworm effect. Moreover, they showed that AMF had no effect and earthworms a positive effect on shoot biomass. Design characteristics could explain these differences. Comparing to Wurst et al. (2004), the duration of our experiment was three times longer and we studied different earthworm and plant species. It has also been shown that AMF influence on

plant growth is plant species dependent and that AMF present a great variety of strategies for P acquisition (Jansa et al. 2005; van der Heijden et al. 1998). Moreover, different earthworm species or ecological categories may variously influence AMF species and plant growth (Brown et al. 2004; Wardle 2002). However, these contradicting results could also highlight that key organisms may be different depending on the nutrient status of the soil, in particular N or P concentration. The plant shoot N:P ratio is generally used to indicate which nutrient limits plant growth. Koerselman and Meuleman (1996) demonstrated that a N:P ratio higher than 16 indicates a P limitation whereas a N:P ratio lower than 14 shows that N is limiting. In our study, we observed a clear P limitation for plants grown without AMF (mean N:P ratio = 22.6; SE = 1.2). In contrast, the N:P ratio evaluated from the paper of Wurst et al. (2004) seems strongly lower that 14 indicating N limitation. We suggest that in P limited conditions, AMF have dominant effects by improving plant phosphorus uptake, whereas in N limited conditions, earthworms can play a major role by enhancing N mineralization (Scheu 1994).

In addition, earthworms interacted significantly with AMF for total C and N soil content. Total C and N were lower in the soil when earthworms were present in the absence of AMF. Scheu (1994) showed that earthworms may enhance N mineralization in the soil thus increasing N uptake and therefore plant growth. We suppose that roots accumulated those mineralized N form, which may explain the lower N content in the bulk soil with earthworms, despite no positive effect of earthworms on total N in the shoots. On the contrary, we measured more total N when both earthworm and AMF were present in the microcosms. It has been previously described by Hawkins et al. (2000) that AMF are able to uptake and transport inorganic N. The analysis made on the bulk soil also contained the hyphal network that cannot be separated. Therefore, we suppose that the higher N content in the bulk soil may be explained by the presence of the hyphal network in the sample. Furthermore, N previously mineralized by earthworms could have been accumulated in the mycorrhizal hyphae.

The significant interactions between AMF and earthworms on total root biomass showed that the presence of earthworms reduced the positive effect of AMF on root biomass. Previous work on the interaction between AMF and Collembola showed that these Insects may reduce plant biomass by grazing on hyphae and spores of AMF (Endlweber and Scheu 2007; Warnock et al. 1982). Despite no effect on hyphal length density, earthworms may have disrupted and disconnected the external hyphae from the plant. The beneficial AMF effect that we observed in our experiment was reduced and therefore the root biomass was lower when both earthworms and AMF were present.

As described by several authors, the amount of nutrients was significantly different between the three soil compartments. In accordance with the study of Decaens et al. (1999), drilosphere soil (DS) contained more total N but less total C than the bulk soil. According to the results of Le Bayon and Binet (2006), we measured a higher P availability in the DS compared to the bulk soil. However, despite the presence of higher amounts of P and N in the DS, shoot or root biomass was not enhanced when no AMF was added. We suggest that the nutrients contained in the DS may be potentially temporarily stored in burrow walls acting thus as a sink of elements. Another hypothesis is that nutrients

are not directly accessible to roots due to either a low amount of available nutrient forms or to a low number of macropores accessible to leek roots. The significant negative effect of AMF on P availability in the DS would therefore confirm the extensive role of the mycelium network system that may colonize the drilosphere compartment, thus providing a nutrient resource.

Conclusion

Under P limitation, our study demonstrated that earthworms and AMF differently affected soil parameters and plant growth. We principally observed an effect of earthworms on soil physical properties, of AMF on chemical properties and of plant roots on both physical and chemical properties. In contrast with previous studies that mainly focused on plant performance or plant nutrient uptake (Ortiz-Ceballos et al. 2007; Smith et al. 2004; Sorensen et al. 2008), we also performed soil parameter measurements. By measuring those parameters we were able to highlight the importance of physical and chemical soil properties to better understand interactions between soil organisms and to interpret contradicting or unexpected results. When studying belowground biotic interactions, future prospects should therefore better take into account chemical and physical soil parameters.

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3

Impact of roots, earthworms and mycorrhizae on soil phosphorus (P) distribution and plant growth as influenced by P fertilization

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In preparation

Abstract

Phosphorus (P) is an essential plant nutrient, but mostly unavailable for plant uptake. Soil organisms are very important for regulating nutrient P cycling. We set up a compartmental greenhouse experiment to study the effects of earthworms (*Allolobophora chlorotica*), arbuscular mycorrhizal fungi (AMF, *Glomus intraradices*) and leek plants (*Allium porrum*) on P distribution (total, organic, and available) and phosphatase activity in the bulk soil, the drilosphere soil (the soil fraction directly influenced by earthworms) and the rhizosphere soil (the soil fraction directly influenced by roots) in presence or absence of P fertilization. P fertilization increased available P in the soil and consequently plant biomass. However, AMF, earthworms or their interaction did not affected soil P distribution or phosphatase activities, despite a higher phosphatase activity and available P in the drilosphere and rhizosphere soil. As a result, plant performance has not been improved in the presence of AMF and earthworms. There is consequently no evidence that the mentioned organisms enhance P uptake in our experiment, even in low P availability in soils.

Keywords

Phosphatase activity; phosphorus; P fertilization; soil interactions; drilosphere; rhizosphere

Introduction

Among soil nutrients, phosphorus (P) is limiting for plant growth, mainly because of its poor mobility in soil (Hinsinger 2001). In contrast to nitrogen, most of the P in the soil is in a range of insoluble inorganic forms, unavailable for plant uptake. In consequence of this low P availability for plants, P fertilizers have been introduced in order to improve plant productivity (Frossard et al. 2004). However, in concomitance with agriculture intensification, P greatly accumulated in soil surface horizons and was largely lost through surface runoff leading to eutrophication (Sharpley et al. 2001). Nowadays, the input of P fertilizers is thought to be reduced in order to better equilibrate optimal plant production while limiting environmental damages (Frossard et al. 2004; Sharpley et al. 2001).

In the soil, total P (P_t) is composed of inorganic and organic forms. The organic phosphorus (P_o) is very abundant in soils and represents between 30% and 65% of the total P (Harrison 1987 in Turner 2008). P_o is dominated by phosphate ester bonds that bind P to C in organic matter. These bonds can be cleaved by phosphatase enzymes, releasing inorganic phosphate (P_i) that can be taken up by the plants (Turner 2008). In comparison with P_o, the P_i represents only a small fraction of the P_t in soils. Phosphate is the main inorganic form of P available for plants. It is generally divided into two forms, labile P and occluded P. The labile pool constitutes the orthophosphate ions (mainly $H_2PO_4^-$ and HPO_4^{2-}) either in soil solution or weakly sorbed onto the soil matrix. It is generally assumed that the amount of labile P in soil is very low, due to the rapid fixation and strong retention (adsorption) of P onto soil constituents bearing positive charges as hydroxyl (Fe and Al oxides), carboxyl (organic matter) or silanol (clays) groups (Hinsinger 2001). The occluded P pool is characterized by P that precipitates with metal cations as Fe and Al in acid soils or Ca compounds in neutral or alkaline soils and becomes insoluble and unavailable to plants.

As a result of the deficiency of available P in the soil, plants developed different strategies to acquire P. These include the extension of the root network, the secretion of organic acids, the synthesis of phosphatase enzymes, the increased secretion of high affinity P–transporters in the cell membranes and/or the formation of proteoid roots. Other soil organisms as arbuscular mycorrhizal fungi (AMF) and earthworms are also known to play a major role in increasing the levels of P availability in soils (Bardgett 2005). It is generally assumed that AMF, considered as an extension of the root network, increase the mobilization of available P by accessing soil P that is beyond the rhizosphere, i.e. the soil fraction directly influenced by the roots (Smith and Read 1997). The mechanisms involved are the secretion of organic anions, mainly oxalates, which increase weathering rates of P contained in clay minerals or the shift of the adsorption-desorption equilibrium towards enhanced desorption (Hinsinger 2001). As for plant roots and bacteria, it has also been demonstrated that AMF were able to produce extracellular phosphatase (Koide and Kabir 2000). Finally, earthworms, as ecosystem engineers, are also known to beneficially affect plant growth via mineralization of nutrients (Edwards and Bohlen 1996; Scheu 2003). It has been shown that P availability was much greater in casts and/or burrows (i.e. drilosphere soil) than the surrounding soil (Le Bayon and Binet 2006; Sharpley and Syers 1976). The ingestion and thorough mixing of soil in the intestinal tract

of earthworms is assumed to influence the dissolution of phosphate rock (Mackay et al. 1982) as well as phosphatase activity (Satchell and Martin 1984; Vinotha et al. 2000).

Studying belowground interactions and particularly the interacting effect of functionally dissimilar soil organisms as AMF and earthworms is of growing interest. In a previous study performed in climate chamber and in P-deficient conditions, AMF but not earthworms affected plant growth (Milleret et al. 2009). However contradicting results were reported when considering the effects of these two soil organisms on plant performance (Eisenhauer et al. 2009; Smith et al. 2004; Wurst et al. 2004). Milleret et al. (2009) suggested that limiting nutrients in soils, mainly N and P, may explain these contrasting results. Moreover, as demonstrated by Zhang et al. (2004), available phosphate concentrations in soil are enhanced by P fertilization. It is therefore supposed that the addition of P fertilizers in P-deficient soils may help better understanding the effect of AMF and earthworms on plant performance.

The aim of the present study is consequently to assess the single or interacting effects of earthworms (*Allolobophora chlorotica*, Savigny), AMF (*Glomus intraradices*, Schenk & Smith) and leek plants (*Allium porrum*, L.) on P distribution and phosphatase activity in the bulk soil, the drilosphere soil (the part of the soil influenced by earthworms) and the rhizosphere soil (the part of the soil influenced by the roots). In comparison with the study of Milleret et al. (2009), the experiment was conducted i) in the presence or absence of P fertilization and ii) under glasshouse condition, i.e. at an intermediate level between climate chamber and field conditions. The effects of earthworms and AMF on plant growth and P content were in turn investigated. In the case of P addition, our working hypotheses were that P fertilization would 1) improve phosphorus concentration in soil, mainly available P that would in turn enhance plant growth, 2) reduce the symbiosis (i.e. the mycorrhization rate) and 3) decrease phosphatase activities. In addition, we suppose that earthworms and AMF would have no influence on P dynamics (i.e. its availability in soil and its further absorption by plants).

Inversely, without P addition, we suppose that 1) the mycorrhization rate by AMF would be increased, 2) AMF and earthworms would impact the P dynamics and phosphatase activities, and 3) they would both enhance plant growth through P uptake. In all cases, we suppose that the soil fractions influenced by earthworms and roots (i.e. the drilosphere and rhizosphere soils) would be beneficial for soil fertility (i.e. accumulation of available nutrients, higher phosphatase activity) compared with the bulk soil.

Material and methods

Experimental setup

The same compartmental microcosm design as described in Milleret et al. (2009) was used. Soil microcosms were separated vertically into two equal parts by a nylon mesh (25 µm, ©SEFAR, Switzerland) and filled with six successive 5 cm thick layers of soil remoistened at 22% (w:w) water content and a bulk density of 1.15 g cm^{-3}. The soil contained 521 µg g^{-1} total phosphorus (P_t), of which 462 µg g^{-1} were in organic forms (88%) and 32.2 µg g^{-1} (6%) were in available forms (mainly $H_2PO_4^-$ and HPO_4^{2-}). In order to eliminate AMF from the soil, microcosms were first sterilized with γ-irradiation (between

42 kGy and 82 kGy; Studer Hard, Dänikon, Switzerland) and stored at 4°C (McNamara et al. 2003). Thereafter, in each microcosm, a 20 ml soil suspension (100 g of soil dispersed in 1000 ml of autoclaved distilled H_2O and filtered on 11 µm paper) was added to re-inoculate the sterilized soil with microorganisms, but without AMF (Koide and Li 1989).

We defined treatments representing all possible combinations of the presence/absence of the four following factors: (1) P fertilization (under the form KH_2PO_4 5mM) (2) three leek plantlets: *Allium porrum* var. Mercure, sown in sterilized conditions, (3) AMF inoculum: 30 g of culture sand substrate mixed with *Glomus intraradices* spores and hyphae (the treatments without AMF received 30 g of a sterilized inoculum, autoclaved at 121°C over 1 h and gamma-irradiated) and (4) five endogeic earthworms: *Allolobophora chlorotica* of equal size and total biomass of 1.3 g (± 0.1 g). Earthworms were relieved of their gut contents before their introduction into the microcosm. The nylon mesh, separating the microcosm into two parts, retains the roots but allows hyphae to pass through. Therefore our compartmental design permitted to separate the individual effect of AMF from the root effect.

The four factors were allocated to the microcosms in three steps. First, Leek plants (L) were attributed randomly on one side of each microcosm. Each microcosm contained therefore both levels of the plant factor (absence/presence). Second, the four possible combinations of AMF (A) and Earthworm (E) factors (A, E, A+E and Control) were randomly allocated to the microcosms. For the microcosms receiving the AMF treatment, 30 g of inoculum was added before introducing the leek plantlets in one side of each microcosm. However, the AMF could colonize both sides by passing through the nylon mesh. For the microcosms receiving the earthworm treatment, groups of five earthworms of equal biomass were prepared and added to both sides of the microcosms. This corresponds to a density of 150 g m^{-2} which is 1.5 times higher than in a maize crop according to Le Bayon and Binet (1999). Finally, the P fertilization factor was randomly attributed to the microcosms. The combination of the four factors gave a total of 16 treatments using eight microcosms (two treatments per microcosm). All the microcosms were replicated three times resulting in a total of 24 microcosms (i.e. 48 samples).

All microcosms were kept in a glasshouse at the botanical garden of Neuchâtel in buffered light and temperature conditions. Soil watering was controlled, microcosms being watered twice a week with a modified Hoagland's nutrient solution with or without P according to the P treatment. Every three weeks, each microcosm was adjusted to equal soil water content with deionised water by weighing. Complete destruction of the microcosm was performed after 45 weeks.

Harvesting and measurements

After 45 weeks, leek shoots were cut at ground level, pooled, weighed and air-dried. Three different soil fractions were removed from the microcosms: (1) Rhizosphere Soil (RS), corresponding to the soil still adhering to the roots after gentle shaking, was collected by rubbing roots carefully on a 2 mm mesh sieve; (2) Drilosphere Soil (DS) was obtained by sampling faeces and the few millimetres-thick layer around the earthworm burrow walls; (3) the remaining Bulk Soil (BS) was thoroughly mixed. Soil samples were air-dried before analyses were performed, except for

phosphatase activity measurements where the soil was stored at 4°C and used in the next two days. After rhizosphere soil collection, roots were carefully washed, mixed, weighed and stored at 4°C in a lactoglycerol-mix made-up of lactic acid/glycerol/ deionised water (1:1:1). Earthworms were hand-collected, counted and weighed.

Mycorrhiza analysis

To measure AMF root infection, roots were first cleared in 10% KOH, acidified in 1% HCl and stained in 0.05% Trypan blue in lactoglycerol. The AMF colonisation was determined on 150 root segments at 250x magnification using a modified line intersect method (McGonigle et al. 1990).

Chemical analysis

After a Kjeldahl oxidation, total P (P_t) concentration was determined colorimetrically at 880 nm using the molybdate procedure (Murphy and Riley 1962) on 0.5 g of pulverised shoots or roots and on 1 g of soil. In addition soil samples were measured for total organic P (P_o). P_o was determined colorimetrically (see above) after combustion at 550°C and digestion in sulfuric acid (H_2SO_4 0.5N) of 1 g of soil. Total inorganic P (P_i) was calculated as the difference between P_t and P_o. In parallel, soil samples were also measured for available P (P_a), a fraction of the inorganic P. According to Olsen et al. (1954), P_a was extracted from samples of 2.5 g of soil with sodium bicarbonate $NaHCO_3$ (0.5 N, pH 8.5) and determined at 880 nm using the Murphy and Riley method (1962).

Enzyme activities

Phosphatase activity was determined according to the method of Tabatabai and Bremner (1969), at pH 5.2 (acid) and pH 10 (alkaline). Acid phosphatase was chosen according to Joner and Johansen (2000) who showed the maximum enzyme activity of the external hyphae of *G. intraradices* at pH 5.5. In addition, alkaline phosphatase was chosen according to Satchell and Martin (1984), who described two peaks of activity at pH 3-5 and pH 9-10 in the presence of earthworms. To 0.5 g of fresh soil, 0.25 ml toluene, 4 ml modified universal buffer (pH 5.2 or pH 10), and 1 ml of p-nitrophenyl phosphate (in modified universal buffer) solution were added and the samples were incubated for 1h at 37°C. The formation p-nitrophenol was determined colorimetrically at 410 nm and results were expressed as mg p-nitrophenol (g^{-1} dw h^{-1}).

Statistical analysis

All the statistical analyses were performed with R 2.6.0 (R Development Core Team 2007). Normal distribution and homogeneity of variance were improved by log-transformation, if necessary, but non transformed means are represented in text and figures (± SE). First, we analysed the effect of the treatments in the bulk soil. For variables with only one measurement per microcosm (shoot and root weights and AMF root colonization), two or three-way ANOVAs were performed with earthworms (E), P fertilization (P) and/or AMF (A) as factors. When both sides of the microcosm were concerned (bulk soil analysis), partly nested ANOVAs were performed in order to take into account the fact that two samples (with or without plants) were in the same microcosm. In this case, leek was considered to

be nested within the microcosm. Consequently, the ANOVA model contained earthworms, AMF and P fertilization as between-microcosm factors and leek as within-microcosm factors. In a second step, we focused on the soil fractions. We therefore created a new dataset with samples containing drilosphere, rhizosphere and bulk soil (i.e. from E+A+L or E+L treatments). Three-way ANOVAs were performed with P fertilization (P), AMF (A) and soil fraction (S) as factors. Partly nested ANOVAs were performed in order to take into account three soil fractions in the same microcosm. In this case, soil fraction was considered to be nested within the microcosm. Consequently, the ANOVA model contained P fertilization and AMF as between-microcosm factors and soil fraction as within-microcosm factors.

Results

Soil organisms

Throughout the experiment, no AMF colonization was observed in the non-AMF treated samples (data not shown). In every case, earthworms colonized the entire microcosm (a lot of burrows observed when sampling). The presence of earthworm did not modify the mycorrhization rates of the plants ($F_{1,8}$= 0.05, P = 0.83), while the mycorrhization rate of plant roots was significantly lower with P fertilization (26 ± 10%) than without P fertilization (78 ± 5%, $F_{1,8}$= 17.36, P = 0.01). A total of 89 of the 120 earthworms (75%) added to the microcosms survived the 45-week experiment. Survival of earthworms was not affected by P fertilization ($F_{1,17}$= 0.29, P = 0.60) and the presence of AMF ($F_{1,17}$= 0.01, P = 0.92), but it was significantly higher in microcosms without plants (120 ± 30%) than in those with plants (28 ± 5%, $F_{1,17}$= 7.11, P = 0.03). However, visual observation of the soil column after removal of the pot indicated that the whole column was burrowed, with burrows reaching the bottom of the column. As for survival, both P fertilization ($F_{1,17}$= 0.83, P = 0.39) and the presence of AMF ($F_{1,17}$= 0.64, P = 0.45) did not affect the fresh body weight of total earthworms. By contrast, the presence of plants significantly affected earthworm biomass ($F_{1,17}$= 6.15, P = 0.04). Earthworms lost weight in the presence of leek plants (21.9 ± 4.3% of initial total body weight) compared with microcosms without plants (97.6 ± 29.3% of initial total body weight).

Plant biomass and P concentration in shoots and roots

P fertilization significantly increased the total plant biomass, as well as the root and shoot biomass (Table 3.1). In the absence of AMF and earthworms, plant biomass was 3.7x heavier than unfertilised plants. Contrarily to our expectation, AMF, earthworms or their interaction had no effect on the leek biomass. P concentration in the roots and shoots was also significantly higher with P fertilization (3.3x for roots and 1.5x for shoots). In addition P concentration was significantly higher in the presence of AMF for both roots and shoots, whereas P concentration in the shoots was lower in the presence of earthworm.

Phosphorus distribution in the soil

Bulk soil analysis

The measured P concentrations in all fractions, except P_o, were significantly higher with P fertilisation (Table 3.2). P_t concentration in the bulk soil was 1.2 times, P_i 1.6 times and

Table 3.1 Mean values (± SE) of biomass (g dried weight), phosphorus (P) concentration (mg g^{-1}) in the roots, shoots and total plants. Significant effects are given in bold. ↑ = increase; ↓ = decrease.

			Root		Shoot		Total
	A	E	biomass	P concentration	biomass	P concentration	biomass
with P							
	-	-	3.91 (0.32)	6.09 (0.50)	50.46 (3.46)	3.03 (0.14)	54.37 (3.69)
	-	+	4.44 (0.33)	5.25 (0.62)	46.58 (4.37)	3.02 (0.09)	51.02 (4.65)
	+	-	3.28 (0.37)	6.22 (0.58)	47 97 (6.27)	3.46 (0.34)	51.24 (6.59)
	+	+	4.72 (1.39)	6.13 (0.65)	45.17 (7.93)	3.00 (0.07)	49.88 (9.26)
without P							
	-	-	1.52 (0.34)	1.52 (0.09)	13.08 (1.70)	2.24 (0.24)	14.59 (2.00)
	-	+	1.79 (0.29)	1.23 (0.16)	18.53 (1.86)	1.36 (0.16)	20.32 (2.03)
	+	-	1.30 (0.38)	2.44 (0.20)	16.87 (2.65)	2.33 (0.03)	18.17 (3.02)
	+	+	1.38 (0.25)	1.95 (0.15)	17.12 (1.36)	2.18 (0.19)	18.50 (1.61)
ANOVA *P*-value							
P			**<0.001** ↑	**<0.001** ↑	**<0.001** ↑	**<0.001** ↑	**<0.001** ↑
A			0.55	**0.04** ↑	0.90	**0.04** ↑	0.85
E			0.17	0.17	0.94	**0.02** ↓	0.92
P x A			0.87	0.60	0.60	0.39	0.66
P x E			0.33	0.91	0.31	0.34	0.43
A x E			0.66	0.66	0.73	0.63	0.80

Table 3.2 Partly nested ANOVA showing the effects of P fertilization, earthworms, AMF and leek roots on total, organic, inorganic and available phosphorus and on acid (pH 5.2) and alkaline (pH 10) phosphatase activity in the bulk soil. P: P fertilization, E: Earthworms, A: AMF, L: leek plants, df degrees of freedom, MS mean square, ns not significant. P< 0.1, *P<0.05, ** P<0.01, *** P<0.001, ↑ = increase; ↓ = decrease.

		Phosphorus (P) fractions									Phosphatase activity				
	df	P_t			P_o			P_i			P_a			acid			alkaline		
		F	*P*		F	*P*		F	*P*		F	*P*		F	*P*		F	*P*	
Between microcosms																			
P	1	180.44	***	↑	<0.01	ns		77.18	***	↑	788.51	***	↑	1.62	ns		3.18	.	(↑)
E	1	0.01	ns		1.69	ns		0.60	ns		0.86	ns		2.55	ns		0.20	ns	
A	1	3.05	.	(↓)	0.76	ns		1.80	ns		0.02	ns		0.01	ns		3.32	.	(↑)
P x E	1	1.49	ns		0.34	ns		0.55	ns		2.11	ns		0.20	ns		5.66	*	
P x A	1	4.06	.		0.10	ns		0.11	ns		<0.01	ns		0.02	ns		0.16	ns	
E x A	1	4.35	.		0.37	ns		1.56	ns		3.32	.		2.52	ns		0.66	ns	
Residuals (MS)	17	1898			0.01			5171			173			0.02			0.01		
Within microcosms																			
L	1	4.12	.	(↓)	13.48	**	↓	0.58	ns		1.55	ns		11.05	**	↑	10.46	**	↑
P x L	1	0.55	ns		0.49	ns		0.50	ns		0.06	ns		3.40	.		0.19	ns	
L x E	1	2.03	ns		5.01	.		<0.01	ns		2.24	ns		0.11	ns		1.25	ns	
L x A	1	0.03	ns		2.12	ns		0.45	ns		1.30	ns		3.99	.		1.47	ns	
Residuals (MS)	20	2288			<0.01			3598			111.97			<0.01			<0.01		

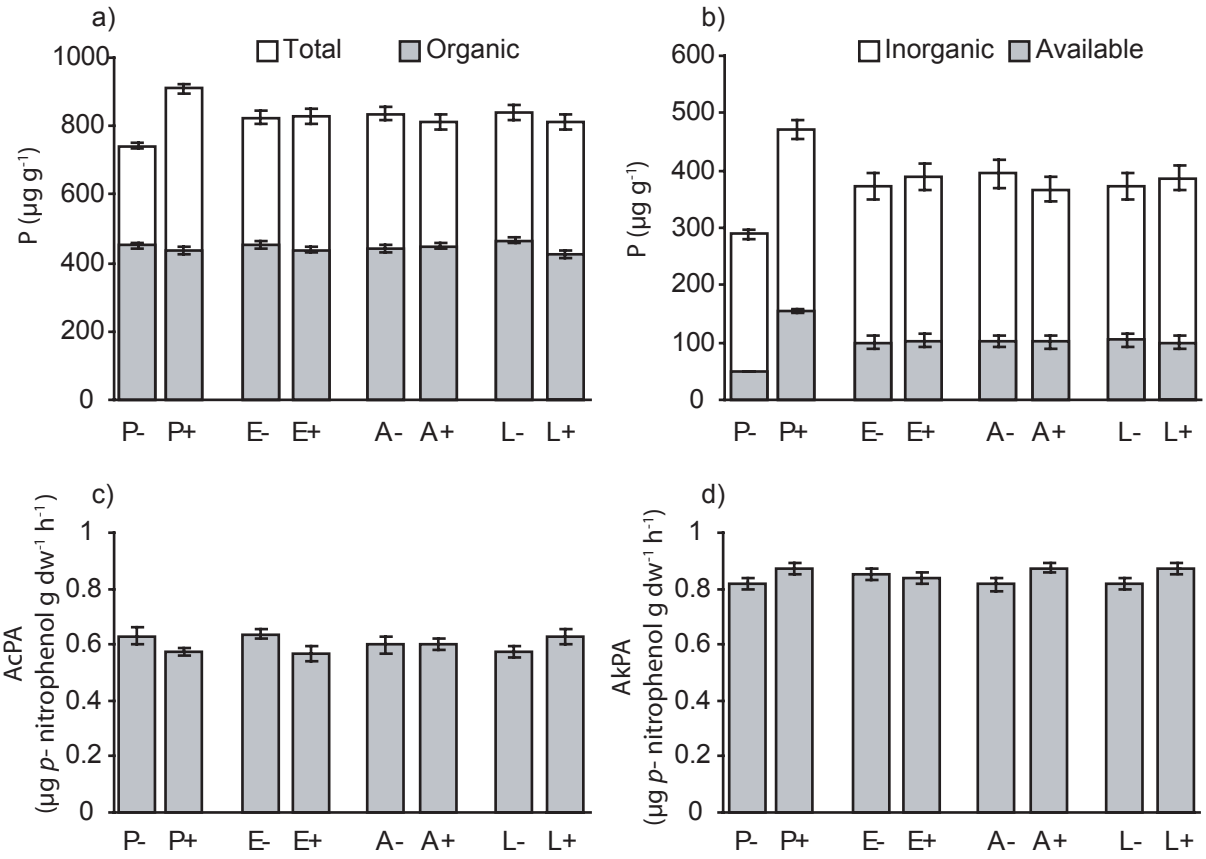

Fig. 3.1 Main effects of P fertilization (P), Earthworm (E, *Allolobophora chlorotica*), AMF (A, *Glomus intraradices*) and Leek (L, *Allium porrum*) on a) total and organic P, b) inorganic and available P, c) acid phosphatase (AcPA, pH 5.2) and d) alkaline phosphatase (AkPA, pH 10). Bar represents mean ± SE.

P_a 3.2 times higher with P fertilization (Fig. 3.1a, b). The proportion of available P in the P_i was doubled with P fertilization. The presence of AMF or Leek only tended to decrease total P, whereas P_o concentration was significantly lower with the presence of Leek in the bulk soil (Fig. 3.1a).

Soil fraction analysis

When analysing the three soil fractions, similar results as those obtained with the bulk soil were observed. P fertilization significantly affected total P, P_i and available P (Table 3.3). In all three cases, P fertilization increased P concentrations, up to 1.9 times with Pi (Fig. 3.2a, b). The presence of AMF had no significant effect on P concentration in any fractions, except that available P tended to be lower with AMF in the microcosm. Total P, P_i and P_a concentrations were significantly affected by the three soil fractions in the following manner: drilosphere ≥ rhizosphere > bulk soil (Fig. 3.2a, b). In addition, P_t and P_a were significantly affected by the interaction between P fertilization and soil fraction (P x S) and P_a by the interaction between soil fraction and AMF (S x A) (Table 3.3).

Phosphatase activity in the soil

Bulk soil analysis

In the bulk soil, phosphatase activity was mainly affected by the presence of Leek (Table 3.2). Both alkaline (pH 10) and acid (pH 5.2)

Table 3.3 Partly nested ANOVA showing the effects of P fertilization, AMF, leek and soil fractions (drilosphere, rhizosphere and bulk soil) on total, organic, inorganic and available phosphorus, and on acid (pH 5.2) and alkaline (pH 10) phosphatase activity. P: P fertilization, A: AMF, S: soil fractions (drilosphere, rhizosphere and bulk soil), df degrees of freedom, MS mean square, ns not significant. . P< 0.1, *P<0.05, ** P<0.01, *** P<0.001, ↑ = increase; ↓ = decrease.

		Phosphorus (P) fractions									Phosphatase activity					
	df	P_t			P_o			P_i			P_a			acid			alkaline		
		F	*P*		F	*P*		F	*P*		F	*P*		F	*P*		F	*P*	
Between microcosms																			
P	1	420.69	***	↑	0.55	ns		376.02	***	↑	2839.62	***	↑	6.08	*	↓	1.51	ns	
A	1	1.62	ns		<0.01	ns		0.88	ns		4.82	.	(↓)	0.02	ns		5.39	*	↑
P x A	1	1.72	ns		1.67	ns		<0.01	ns		1.23	ns		0.09	ns		0.28	ns	
Residuals (MS)	8	<0.01			0.01			0.01			81.00			0.05			0.01		
Within microcosms																			
S	2	23.21	***		3.17	.		5.78	*		31.93	***		17.53	***		11.43	***	
P x S	2	5.57	*		1.05	ns		1.77	ns		50.81	***		3.23	.		9.42	**	
A x S	2	1.05	ns		0.59	ns		0.06	ns		3.87	*		0.61	ns		0.93	ns	
Residuals (MS)	18	<0.01			0.02			0.02			0.01			0.04			<0.01		

phosphatase activity was higher with Leek and the former tended to be higher with AMF and P fertilization (Fig. 3.1c, d). No significant effect of earthworm was observed for both analyses in the bulk soil, except a significant interaction between P fertilization and earthworms (PxE) at pH 10.

Soil fraction analysis

Phosphatase activity was significantly different in the three soil fractions (Table 3.3). In every case, the lowest phosphatase activity was measured in the bulk soil (fig. 3.2 c, d). In addition, phosphatase activity at pH 5.2 was significantly lower with P fertilization, whereas at pH 10 it was higher with the presence of AMF (Fig. 3.2 c, d). At pH 10, phosphatase activity was affected by the interaction between P fertilization and the soil fraction (PxS) and this interaction was marginally significant for pH 5.2.

Discussion

Soil organisms

The percentage of root colonization by AMF is known to vary according to the nutrient availability in the soil as available phosphorus or to other interacting species as earthworms or Collembola (Warnock et al. 1982). When considering earthworms, contradicting results are present in the literature. Some authors suggest a positive effect of earthworms on mycorrhization rate by increasing spore dispersal (Gange 1993; Reddell and Spain 1991), but negative impacts are also reported as earthworms may mechanically damage the AMF mycelium or digest fungal spores (Ortiz-Ceballos et al. 2007; Tuffen et al. 2002). Overall, in the present experiment, we found no effect of the earthworm presence on the mycorrhization rate, which is in accordance with the results of Eisenhauer et al. (2009) and Wurst et al. (2004). On the contrary, the

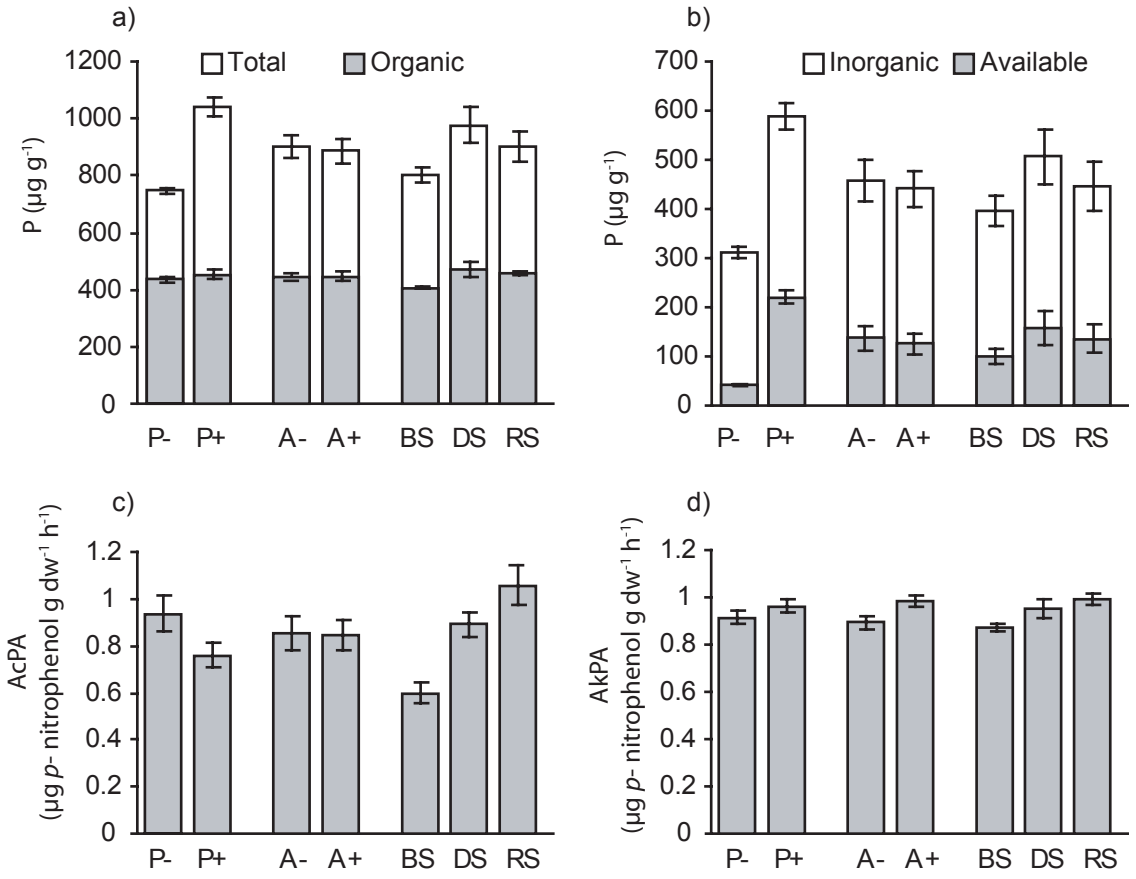

Fig. 3.2 Main effects of P fertilization (P), AMF (A, *Glomus intraradices*) and soil fractions (BS Bulk soil, DS Drilosphere soil, RS Rhizosphere soil) on a) total and organic P, b) inorganic and available P, c) acid phosphatase (AcPA, pH 5.2) and d) alkaline phosphatase (AkPA, pH 10). Bar represents mean ± SE.

mycorrhization rate was strongly affected by P fertilization. The present result is in accordance with previous studies showing a decrease in mycorrhizal root colonization or a reduction of the external hyphal length when P_a is present in sufficient quantity for the roots (Amijee et al. 1989; Bruce et al. 1994; Marschner and Dell 1994; Nogueira and Cardoso 2007).

The presence of leek plants decreased earthworm survival and biomass, as previously described in Milleret et al. (2009) by using a similar compartmental design but in climate chamber. As recently demonstrated by Eisenhauer et al. (2009), litter quantity and quality is highly important for earthworm survival. In particular, the authors highlighted the positive effect of the legume *Trifolium repens* compared with *Plantago lanceolata* on earthworm performance. They suggest that *P. lanceolata* provides dead root material and root exudates of lower quality or quantity than *T. repens*. It is therefore likely that leek root exudates are unbeneficial for earthworm survival, in confined pot experiment at least. Contrarily to leek roots, the presence of AMF and P fertilization did not affect earthworm survival.

P fertilization positively affected plant growth and P concentration in shoots and roots. Total plant biomass was up to 3.7 times greater after 45-week experiment with P fertilization in comparison with unfertilised pots. Using leek plants, Amijee et al. (1989) also found a greater biomass of leek plants with P fertilization (with

a peak at 450 mg kg^{-1}) compared with treatment without P. Despite a significant positive effect of AMF on P concentration in the root and shoot, no effect was noticeable when considering plant biomass. This result is in contradiction with our hypothesis and the results of Milleret et al. (2009) performed in climate chamber, where a strong positive effect of AMF was observed on plant growth. In the present study, without P addition, shoots had a similar biomass independently of the AMF treatment, whereas in the study of Milleret et al. (2009), after 35-week experiment, fresh shoots were more than three times heavier with the presence of AMF than without. Three reasons may explain the result. First, the study of Milleret et al. (2009) was performed in a climate chamber with light intensity varying between 3'000 and 8'000 lux. Combined with P-deficient irrigation, plants should have therefore highly benefited from the presence of AMF. Second, during the present glasshouse experiment, spider mite infection occurred during the second half of the experiment. Despite the utilization of black soap diluted in water to stop the attack, we supposed that unfertilized plants were weaker and suffered from the mite infestation, independently of the presence of AMF. Third, as unfertilized plant roots were still highly mycorrhized (around 70%) after 45 weeks, we suppose that AMF infection was an energetic cost for the plant that could in turn not invest for its own biomass.

Phosphorus distribution in the soil

Most of the studies trying to investigate the interacting effect of AMF or earthworms in the soil focused on their impact on plant performance as shoot and root productivity and nutrient uptake, mainly N and P (Eisenhauer et al. 2009; Ortiz-Ceballos et al. 2007; Tuffen et al. 2002; Wurst et al. 2004). Only few studies consider the impact of these organisms on the soil nutrient dynamic or the availability of these nutrients in the soil. In a previous study, we highlighted the importance of measuring soil parameters for a better understanding of the effects and interactions between soil organisms on plant performance (Milleret et al., 2009). In the present study, and with P fertilization, we measured in the bulk soil a higher concentration of total P, as well as available forms of P within the inorganic pool. This was beneficial for plant growth (see above), but no effect of AMF or earthworms was measured. With P fertilization, we supposed that P was in excess and that no AMF or leek effect could have been observed. However, as in our previous experiment and according to previous studies (Jakobsen et al. 1992; Marschner and Dell 1994), we hypothesized an AMF and root effect on P uptake in the unfertilized treatments. Again, we suppose that it was a consequence of the spider mite infection. Damaged plants without P fertilizers were too weak to uptake available P in the bulk soil.

Contrarily to the results obtained with the bulk soil (no significant effect of roots or earthworms), P measurements were different in the fractions of soil influenced by roots (rhizosphere) and earthworms (drilosphere). In every case, rhizosphere and drilosphere soil had higher P concentration than the bulk soil. In previous studies, Le Bayon and Binet (2006) and Chapuis-Lardy et al. (1998) found no difference in total P between casts or burrows and non-ingested soil (i.e. bulk soil). However, these authors obtained similar results as ours for organic P, available P and inorganic P. In parallel, Le Bayon et al. (2006) found a reduced amount of available P in the rhizosphere of the white lupin in a microcosm experiment. The

authors attributed the results to the special root structures (cluster roots) formed by the lupin. Indeed, cluster roots are known to exude large amounts of low-molecular-weight organic anions that enhance the availability of P to the plants (Gerke et al. 1994). These contrasting results highlight the importance of soil organism activities to enhance nutrient availability.

Phosphatase activity

Overall, we found a greater activity of alkaline phosphatase than acid phosphatase. This is in accordance with the results of Eivazi and Tabatabai (1977), who showed a greater alkaline phosphatase activity in alkaline soil and a greater acid phosphatase activity in acid soils. Interestingly, in the present experiment, P fertilization had no effect on phosphatase activities in the bulk soil. This is in contradiction with our hypothesis as we had supposed a negative feedback of P fertilization on enzyme activity as previously described by Olander and Vitousek (2000). However, Criquet and Braud (2008) suggest different effect of phosphatase activity according to the kind of P fertilization. They found no effect of P fertilization under the form of Na or K Pi-salts on phosphatase activity contrarily to sewage sludge in a Mediterranean soil. We added KH_2PO_4 in the present experiment; our results are therefore in accordance with the study of Criquet and Braud (2008). Additionally, both acid and alkaline phosphatase activities were significantly affected by the presence of leek and organic P was significantly reduced in the bulk soil. This result confirmed the important role of roots in producing phosphatase enzymes, and more interestingly, their ability to enhance enzyme activities not strictly in the rhizosphere but also in the bulk soil.

When analysing the different soil fractions, both acid and alkaline phosphatase activities were significantly higher in the drilosphere and the rhizosphere soil fractions compared to the bulk soil. They followed the same pattern: bulk soil < drilosphere soil ≤ rhizosphere. This result reflects the importance of root and earthworms-mediated soil fractions on enzyme activities. Higher enzyme activities in casts have been widely described (Kizilkaya and Hepsen 2004; Le Bayon and Binet 2006; Satchell and Martin 1984; Vinotha et al. 2000). This phenomenon was generally accompanied with a higher available nutrient content in casts as demonstrated in the present study for P.

In addition to the effect of soil fractions on enzyme activities, the presence of AMF significantly induced an enhanced alkaline phosphatase activity. This was in accordance with the study of Raiesi and Ghollarata (2006). However, contradicting results reported an increased phosphatase activity with AMF (Joner et al., 2000) or not (Joner and Jakobsen, 1995). In particular, Joner and Jakobsen (1995) measured a lower acid enzyme activity with AMF in the presence or absence of clover leaves, whereas in the same experiment, they measured a higher alkaline phosphatase activity with clover leaves alone. Nevertheless, the authors report a low quantitative importance of extracellular phosphatase of AMF for the P nutrition of AM plants. This may explain the lack of AMF effect on plant biomass in the present experiment.

Conclusion

In the present study, phosphorus distribution was mainly influenced by P fertilization that increased P_i in the soil, leading to a greater plant biomass. Indirectly, we suppose that leek

growth was also enhanced due to the higher phosphatase activity and thus mineralization of P_o in the soil. Contrarily to our expectations, the individual or combined effect of AMF and earthworms did not particularly affected P distribution, plant P uptake or plant growth. The results were particularly obvious in the absence of P fertilization. No AMF effect was observed in the absence of P fertilization, despite a high mycorrhization rate. We therefore suppose that for the plants, the net energetic costs of the symbiosis exceed the net benefits, thus indicating parasitic rather than mutualist interactions between AMF and plants (Johnson et al. 1997). Moreover, despite a higher phosphatase activity and accumulation of available P measured in the soil fractions directly influenced by earthworms (drilosphere soil) and plant roots (rhizosphere soil), plant performance has not been improved. There is consequently no evidence that the mentioned organisms enhance P uptake in our experiment, even in low P availability in soils. However, mechanisms of the interaction between AMF and earthworms are still poorly understood and contrasting results are reported in the literature (Ma et al. 2006; Tuffen et al. 2002; Wurst et al. 2004). The only studies aiming at determining the interacting effects of AMF and earthworms are experimental and the length of the experiments is generally short (two or three months). Further field experiment investigating the effects of these organisms within the soil profiles and integrating the P runoff is consequently recommended.

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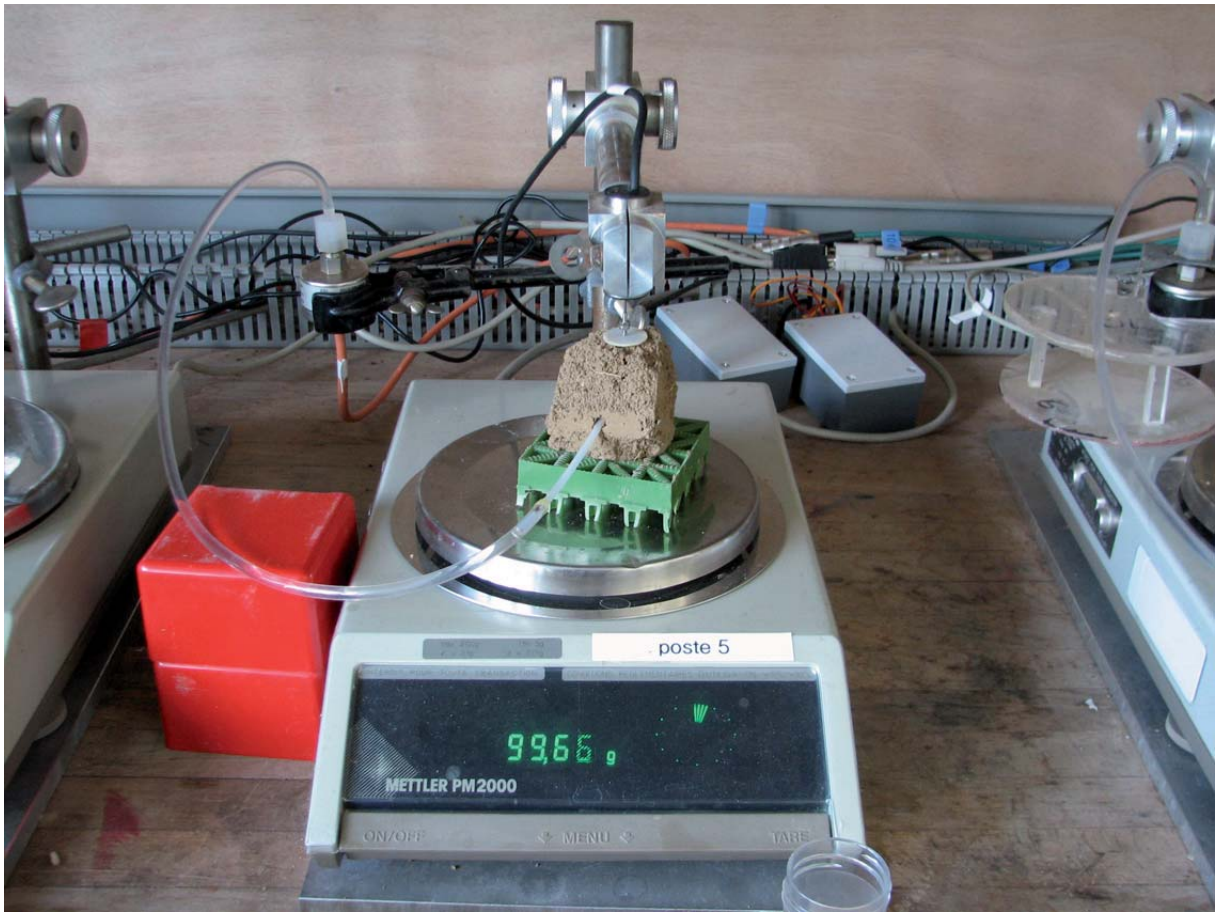
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Impact of roots, mycorrhizas and earthworms on soil physical properties as assessed by shrinkage analysis

Roxane Milleret, Renée-Claire Le Bayon, Frédéric Lamy, Jean-Michel Gobat, Pascal Boivin

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Abstract

Soil biota such as earthworms, arbuscular mycorrhizal fungi (AMF) and plant roots are known to play a major role in engineering the belowground part of the terrestrial ecosystems, thus strongly influencing the water budget and quality on earth. However, the effect of soil organisms and their interactions on the numerous soil physical properties to be considered are still poorly understood. Shrinkage analysis allows quantifying a large spectrum of soil properties in a single experiment, with small standard errors. The objectives of the present study were, therefore, to assess the ability of the method to quantify changes in soil properties as induced by single or combined effects of leek roots (*Allium porrum*), AMF (*Glomus intraradices*) and earthworms (*Allolobophora chlorotica*). The study was performed on homogenised soil microcosms and the experiments lasted 35 weeks. The volume of the root network and the external fungal hyphae was measured at the end, and undisturbed soil cores were collected. Shrinkage analysis allowed calculating the changes in soil hydro-structural stability, soil plasma and structural pore volumes, soil bulk density and plant available water, and structural pore size distributions. Data analysis revealed different impacts of the experimented soil biota on the soil physical properties. At any water content, the presence of *Allolobophora chlorotica* resulted in a decrease of the specific bulk volume and the hydro-structural stability around 25 %, and in a significant increase in the bulk soil density. These changes went with a decrease of the structural pore volumes at any pore size, a disappearing of the thinnest structural pores, a decrease in plant available water, and a hardening of the plasma. On the contrary, leek roots decreased the bulk soil density up to 1.23 g cm^{-3} despite an initial bulk density of 1.15 g cm^{-3}. This increase in volume was accompanied with a enhanced hydro-structural stability, a larger structural pore volume at any pore size, smaller structural pore radii and an increase in plant available water. Interestingly, a synergistic effect of leek roots and AMF in the absence of the earthworms was highlighted, and this synergistic effect was not observed in presence of earthworms. The structural pore volume generated by root and AMF growth was several orders of magnitude larger than the volume of the organisms. Root exudates as well as other AMF secretion have served as carbon source for bacteria that in turn would enhance soil aggregation and porosity, thus supporting the idea of a self-organization of the soil-plant-microbe complex previously described.

Keywords

Shrinkage analysis (ShC), soil porosity, earthworms, arbuscular mycorrhizal fungi (AMF), plant root, soil structure.

Introduction

As an interface between lithosphere and atmosphere, the soils control the earth water budget *via* its physical properties which determine the runoff and infiltration fractions. Water quality is strongly influenced by infiltration through the soil as well. These properties result from the equilibrium between constituents, soil life, and external factors, which vary on different time and space scales. Characterizing and predicting soil physical properties and their changes with time as a function of these factors are essential. Therefore, integrated approaches aiming at better understanding interactions between physical and biological processes in the soil and with the aboveground system are encouraged (e.g. Young and Crawford 2004).

Many authors described soil-biota interactions based on the hierarchical aggregation model developed by Tisdall & Oades (1982) who emphasized the importance of bacteria, fungi and roots in soil aggregation (Brussaard et al. 2007; Dorioz et al. 1993; Feeney et al. 2006; Jastrow and Miller 1991; Six et al. 2004). For example, the physical habitat of soil bacteria in terms of aggregate (Caesar-TonThat et al. 2007; Mummey et al. 2006; Ranjard and Richaume 2001) or porosity (Feeney et al. 2006) was investigated and led to the concept of a *self-organization of the soil-microbe complex* (Young and Crawford 2004). This concept assumes that soil structure initially defines microbial communities but is in turn modified through active microbial activities that alter pore geometry and stability. In addition, roots are known to modify the soil porosity and aggregation via direct entanglement of particles, the creation of biopores or secretion of glue-substances sticking particles together as reviewed by Angers & Caron (1998). Similar to plant roots, AMF are a very important component of the soil system. They influence soil aggregation by binding and enmeshing soil particles into larger aggregates. They also secrete a glycoprotein called glomalin that act as a glue-substance (see Rillig and Mummey 2006 for a review). Earthworms, as ecosystem engineers (Lavelle et al. 1997), play subsequently a very important bioturbation role on soil structure through numerous ways such as casting and burrowing activities, and the transit of the soil through their digestive system, thus promoting the formation of the organo-mineral complex (Brown et al. 2004).

Describing the various effects of soil life on soil physical properties is a challenge. First, because of the numerous properties to be determined, e.g. soil volume or density, soil structure and structural stability, soil pores and aggregate size distributions, water retention curve (WRC), hydraulic conductivity, mechanical properties, each property requiring a specific characterization technique. Second, because the determination must be accurate enough to assess the changes, while most physical properties have a large variability (e.g. Gascuel-Odoux 1987; Nielsen et al. 1973; Sisson and Wierenga 1981; Vauclin 1982).

The recent development of soil shrinkage analysis might overcome these limitations with determining in a single experiment many soil physical properties, namely hydro-structural stability (Schaffer et al. 2008), soil structural and plasma pore volumes, pore volume dynamic with water, soil water holding capacity and water retention curves (WRC) (Boivin et al. 2006a; Braudeau et al. 1999; Braudeau et al. 2004) with small standard errors (Boivin 2007). Soil shrinkage was defined as the soil specific volume change with water content (Haines 1923). It has been long ago used to assess

soil structural stability and soil pore volume (Boivin et al. 2006a; Braudeau et al. 1999; Braudeau et al. 2004). Shrinkage analysis is based on the simultaneous and quasi continuous measurement of soil shrinkage curve (ShC) and WRC (Boivin et al. 2004), and the analysis of the ShC with XP (for exponential) model (Braudeau et al. 1999) or the equivalent PS (for Pedostructure) model (Braudeau et al. 2004). XP/PS models are based on the assumption that there is a dual pore system in the soil (Braudeau 1988a; Braudeau et al. 2004). Fitting XP model equations on an experimental ShC allows determining the volume, air and water content of these two pore systems at any soil water content, and the slopes of the shrinkage domains which can be considered as measures of the soil hydro-structural stability (Schaffer et al. 2008). It has been shown that the two pore systems quantified by shrinkage analysis are the plasma pores and the structural pores, respectively, long ago characterized by micromorphologists (Brewer 1964). Plasma pores are made of the soil colloids (SSSA, 2008) and assumed to shrink like a clay paste, i.e. with no air entry on most of the water content range. The structural pores are made of biopores, lacunar voids between plasma and skeleton, and cracks. Structural pores are assumed to be semi-rigids, hence air entry is partly compensating the loss of water in the structural pores, when the soil is drying.

Shrinkage analysis has been applied to assess the impact of clay content and clay type on soil properties (Boivin et al. 2004), the impact of soil organic carbon on soil physical properties (Boivin et al. 2009), and the impact of trafficking on soil pore properties (Boivin et al. 2006b; Schaffer et al. 2008). Shrinkage analysis, however, has not been used yet to assess the physical impact of soil biota on soil.

The objective of this study was to test the potential of shrinkage analysis to assess changes in soil properties as induced by three model organisms, namely leek roots (*Allium porrum* L.), an arbuscular mycorrhizal fungus (*Glomus intraradices* Schenk & Smith) and an earthworm (*Allolobophora chlorotica* Savigny) in a microcosm experiment.

Material and methods

Experimental setup, plant, mycorrhiza and earthworm

The organo-mineral horizon of an Anthrosol (IUSS 2006) was collected at the botanical garden of Neuchâtel (Switzerland). The soil is a carbonated loamy soil (45.3 % sand, 28.0 % silt and 26.7 % clay), containing 20.7 % (w:w) carbonates, 2.0 % (w:w) total organic carbon and showing a pH_{KCl} of 7.8. The CEC per kg of soil was 21.3 $cmol_c\ kg^{-1}$. A compartmental microcosm design was set up. It consisted of a PVC tube (35 cm height and 15 cm internal diameter) separated vertically into two equal parts by a nylon mesh (25 µm) to separate the individual effect of AMF from the root effect as roots could not pass through the mesh.

The soil was air-dried, sieved to 2 mm size aggregates, homogenized and gamma-ray sterilised (between 42 and 82 kGy) prior to repacking in each side of microcosms with six successive 5 cm thick layers of soil remoistened at 22% water content. Microcosms had a final bulk density of 1.15 g cm^{-3}. Afterwards, a 20 ml soil suspension (100 g of soil dispersed in 1000 ml of autoclaved distilled H_2O and filtered on 11 µm paper) was added to re-inoculate the sterilized soil with microorganisms, but without AMF (Koide and Li 1989).

We applied a factorial design with three factors and one replicate of each treatment. The treatments were all the possible combinations of the presence/absence of three factors, thus involving four repetitions of each factor. These factors were leek (*Allium porrum* var. Mercure, 18 days old, sown in sterilised conditions), AMF (*Glomus intraradices,* 30 g per microcosm of spores and hyphae), and endogeic earthworms (*Allolobophora chlorotica*, five individuals of equal biomass (1.3 g ± 0.1 g) added in each side of the microcosm). This corresponds to a density of 650 individuals m^{-2} which is 3.4 times higher than the density found for a single endogeic species (*Aporrectodea caliginosa*) sampled in a maize crop according to Le Bayon and Binet (1999). We selected an endogeic species because this kind of earthworms inhabit the organo-mineral soil horizon feeding on the soil organic matter closely linked to the mineral matrix; as a matter of fact, they consume more soil than other ecological categories to fulfil their nutritional requirements and consequently largely burrow within the upper centimetres of the soil (Capowiez 2000; Lee and Foster 1991).

The microcosms were kept 35 weeks in a climate chamber under the following conditions: photoperiod 16/8 h (day/night), temperature 18 ± 2 °C, 50% humidity. Irrigation was performed twice a week using a modified Hoagland's nutrient solution without P (Milleret et al. 2009) in order to promote the AMF-plant symbiosis. Every three weeks, each microcosm was weighted and adjusted to equal soil water content with deionised water.

Sampling

After 35 weeks, undisturbed soil cores of approximately 100 cm^3 volume were removed from each side of the microcosm (i.e. with or without roots) for soil shrinkage curve (ShC) and water retention curve (WRC) measurement.

Shrinkage analysis

Quasi-continuous ShC and WRC were determined on undisturbed sub samples of approximately 100 cm^3. The equipment and methods used are the same as presented in Boivin et al. (2004) and Schaffer et al. (2008). Briefly, we wetted the soil samples with deionised water by applying a water potential of –1 kPa with respect to the centre of the samples.

During drying, the samples were placed on electronic balances (0.01 g precision) contained in a thermostatic chamber at 20 °C. Calibrated displacement transducers (resolution of 1 µm) were used to measure changes in sample height during drying. Tensiometers (ceramic cups; length 2.0 cm, diameter 0.2 cm) connected to pressure transducers were inserted in the middles of the samples to measure the water potential (resolution of 1 hPa). Weight, height and water potential were recorded at intervals of 5 minutes until the sample weights reached constant values, which took about 4 days. Then, the dry sample volumes were determined by means of hydrostatic weighing with the plastic bag method described by Boivin *et al.* (1990), and the samples were dried in an oven at 105 °C for 24 hours to obtain the dry weight.

Changes in sample height were converted to changes in specific bulk sample volume by

$$V = V_E \times \left(\frac{H}{H_E} \right)^3, \quad (1)$$

where the exponent 3 denotes isotropic shrinkage (e.g. Boivin 2007), V_E and H_E are the specific bulk volume and height at the end

of the experiment, and *V* and *H* are the bulk volume and height during the experiment.

The XP model equations (Braudeau et al. 1999) were fitted to the experimental shrinkage data by a non-linear simplex method (Chen and Saleem 1986) to determine the coordinates of the transition points between the shrinkage domains (Figure 4.1), namely shrinkage limit (SL), air entry (AE), the dry point of structural porosity (ML), and the maximum swelling of the plasma (MS). The slope of the structural shrinkage domain K_{str} was calculated as:

$$K_{str} = \frac{[V(ML) - V(MS)][\exp(1) - 1]}{[W(ML) - W(MS)] - K_{Bs}[\exp(1) - 2]}, \quad (2)$$

where V_{ML}, W_{ML}, V_{MS} and W_{MS} are the volume and water content of the soil at MS and ML, respectively and K_{Bs} the slope of the basic domain calculated as:

$$K_{Bs} = \frac{V_{AE} - V_{ML}}{W_{AE} - W_{ML}}, \quad (3)$$

where V_{ML}, W_{ML}, V_{AE} and W_{AE} are the volume and water content of the soil at ML and AE, respectively.

Using the XP model equations for the plasma porosity given by Braudeau & Bruand (1993), we then calculated the specific plasma porosity, V_p (in $cm^3\ g^{-1}$ of soil), and the plasma water content, W_p (in $g\ g^{-1}$ of soil). The specific air content of the plasma, A_p, was calculated as

$$A_p = V_p - W_p \ , \quad (4)$$

The specific structural porosity, V_s, was calculated as

$$V_s = V - V_p - \rho^{-1} \ , \quad (5)$$

where ρ^{-1} is the specific volume of the solid phase (set to $1/2.65\ cm^3\ g^{-1}$). The specific water content of the structural porosity, W_s, was calculated as

$$W_s = W - W_p \ , \quad (6)$$

where *W* is the total gravimetric water content.

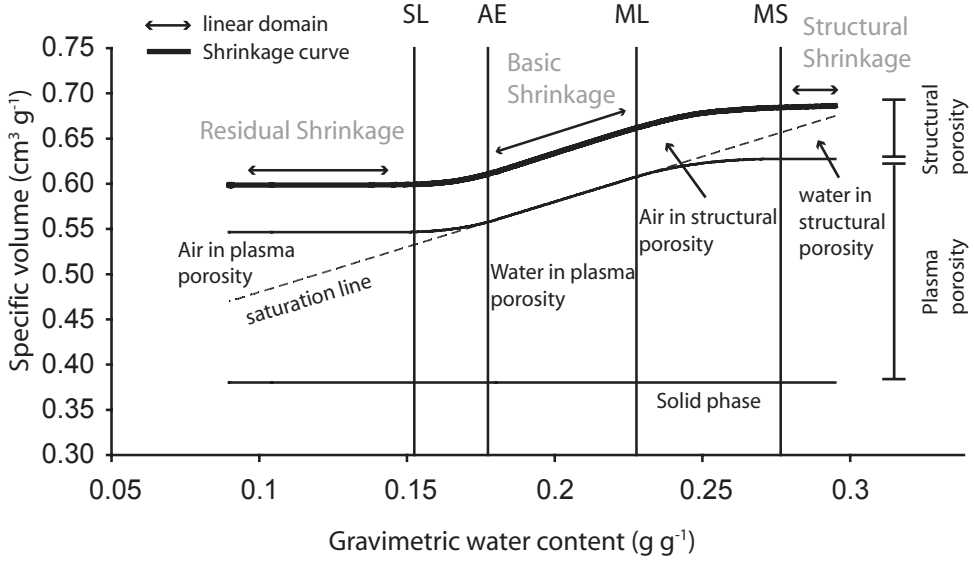

Fig. 4.1 Example of a shrinkage curve with the transition points (SL, shrinkage limit; AE, air entry; ML, macro-porosity limit; MS, maximum swelling), linear domains (residual shrinkage, basic shrinkage, structural shrinkage), and cumulated calculated specific volumes (from bottom to top: solid phase, water in plasma porosity, air in plasma porosity, water in structural porosity, air in structural porosity and bulk soil volume that is shrinkage curve) with saturation 1:1 line.

The specific air content of the structural porosity, A_s, was calculated as

$$A_s = V_s - W_s \text{ , (7)}$$

Bulk density was calculated as the inverse of the specific bulk volume, *V*. Plant Available Water (AW in g g^{-1}) content was determined as the difference between W at MS and AE, and Easily Available Water (EAW in g g^{-1}) was calculated as the difference between W at MS and ML (Braudeau 1988b).

After ShC analysis, the undisturbed samples were broken up to measure the dry root weight in each soil sample (see below, root and AMF size distribution).

Structural pore size distribution

The simultaneous weight and tensiometer measurements were used to determine the water retention curves. Shrinkage analysis allowed calculating the plasma and structural pores water retention curves, by using the W_s and W_p values at any soil water content. We converted the structural pores water retention curves into the structural pore-size distributions of equivalent cylindrical pores using the Jurin–Laplace equation (e.g. Lawrence 1977). Since only the structural pores allow air entry in the tensiometer reading pressure range, we did not apply the procedure to the plasma pores.

Root and AMF size distribution

Specific root volume per class of root diameter (750, 375, 175 and 75 μm) in the microcosms were measured as follows. First, the dried root weight per class of root diameter was measured on the root network remaining in the microcosms after the soil core sampling as described by Blouin et al. (2007). Briefly, the roots were dried at 50 °C and cut in a variable speed rotor mill (Fritsch, Laval lab inc., Canada) with a 2 mm sieve in order to obtain pieces of 2 mm length. Roots were placed on a sieve shaker at continuous agitation for 20 minutes with five successive sieves (1 mm, 0.5 mm, 0.25 mm, 0.1 mm and 0.05 mm).

A potential problem with the employed method is that roots of a smaller size may stick on a larger sieve size because the roots fell horizontally on the sieve. We, therefore, tested the method for the leek root system by visual observation of the root diameter with a binocular. We observed homogeneous root fragments within each class of diameter which allowed us to apply the method of Blouin et al. (2007).

Root fraction in each sieve was then weighted and the ratio of the weight of each root diameter fraction on the total root weight was calculated. This ratio was used to calculate the dry root weight of each root diameter fraction contained in the soil cores from the total root weight of the cores, by taking into account the root weight in the undisturbed samples used for ShC analysis.

In parallel, fresh root fragments were individually weighed, scanned at high resolution and dried at 50 °C overnight. The fresh and dry length, diameter, surface area and volume of the scanned fragments were subsequently calculated using an image analysis program (Image J v.1.40, National Institute of Health, USA). This allowed to determine the following regression ($r^2 = 0.73$, $P<0.001$, n=76) in order to convert dry weight (DW) to dry volume (DV):

$$DV = 2.146\ DW + 0.002 \text{ , (8)}$$

The dry volume was thereafter converted in fresh volume (FV) by using the following equation ($r^2 = 0.79$, $P < 0.001$, n = 76):

$$FV = 1.551\ DV + 0.001\ , \quad (9)$$

The fresh volume per class diameter was finally divided by the soil core weight to have the Specific fresh Root Volume (SRV) per gram of soil ($cm^3\ g^{-1}$) for each class diameter.

The Specific AMF external Hyphal Volume (SHV) per gram of soil was calculated using the hyphal length density (HLD) ($m\ g^{-1}$) measured as described in Milleret et al. (2009). HLD was determined by using an aqueous extraction and a membrane filter technique modified after Jakobsen et al. (1992). Briefly, three replicates of a 4 g soil sample were dispersed in a sodiumhexametaphosphate solution (35 g l^{-1}) and shaken for 30 s (end-over-end). After 30 minutes, the suspension was decanted quantitatively through a 40 µm sieve to retain hyphae, roots and organic matter, transferred with 200 ml of deionised water into a 250 ml flask and shaken vigorously by hand for 5 s. After 1 min, 4 x 1 ml aliquots (10 sec interval) were taken and pipetted onto Millipore RAWG02500 membranes (Millipore, Bedford MA, USA). The filter was finally stained in 0.05% Trypan Blue. HLD was estimated with a gridline intersect method at 250 × magnification (Newman 1966). HLD measurements allowed calculating the length of external hyphae in the cores. Combined with the mean hyphal diameter (15 µm), we therefore calculated the specific AMF external hyphal volume.

Statistical analysis

We performed the statistical analyses with R 2.6.0 (R Development Core Team 2007). We used three-way analyses of variance with the presence/absence of leek roots, AMF and earthworms as independent variables. The effects of independent variables were considered to be significant if the probability of the null hypothesis was ≤0.05.

Results

Biological activity

The quantification of the biological activity is reported in details in (Milleret et al. 2009). Throughout the experiment, no AMF colonization was observed in non-AMF-treated sample. Leek plants were heavier and better developed with AMF and earthworms had a high activity. Earthworms reproduced actively, leading up to 49 individuals in the AMF + Earthworm + Leek treatment. This is ten-fold greater than the five initial earthworms introduced at the beginning of the experiment. Their average biomass increased therefore from 1.32 g to 2.99 g. In the other treatments, the number of earthworms at the end of the experiment varied between 11 and 24 individuals and the mean biomass was similar when comparing the start and the end of the experiment (around 1.3 g). This was explained by the presence of juveniles with a low biomass (about 0.01 g per individual).

Table 4.1 shows the specific fresh root volume (SRV) per gram of soil ($cm^3\ g^{-1}$) per class of diameter as well as the total SRV and the specific AMF external hyphal volume. Roots occupied a total volume ranging from 2.6E-04 to 1.6E-03 $cm^3\ g^{-1}$ of soil and AMF occupied a total volume ranging from 1.1E-03 to 1.5E-03 $cm^3\ g^{-1}$ of soil.

Table 4.1 Specific fresh Root Volume (SRV) per class of diameter ($cm^3\ g^{-1}$), total SRV ($cm^3\ g^{-1}$), Specific AMF external Hyphal Volume (SHV in $cm^3\ g^{-1}$) and air filled pore volume at water saturation ($cm^3\ g^{-1}$) for each of the eight microcosms. SHV was calculated based on the hyphal length density ($m\ g^{-1}$) and the mean hyphal diameter (15 µm).

Leek	AMF	Earthworm	SRV per class of diameter ($cm^3\ g^{-1}$)				Total SRV ($cm^3\ g^{-1}$)	Total SHV ($cm^3\ g^{-1}$)	Air filled pore volume at water saturation ($cm^3\ g^{-1}$)
			750µm	375 µm	175 µm	75 µm			
+	+	+	5.27E-05	9.92E-05	6.01E-05	4.86E-05	2.61E-04	1.13E-03	0.33
+	+	-	4.77E-04	8.31E-04	1.64E-04	1.30E-04	1.60E-03	1.54E-03	0.61
+	-	+	1.58E-04	2.56E-04	1.70E-04	2.46E-04	8.31E-04		0.33
+	-	-	1.01E-04	4.01E-04	2.06E-04	2.21E-04	9.28E-04		0.48
-	+	+						1.25E-03	0.10
-	+	-						1.05E-03	0.37
-	-	+							0.07
-	-	-							0.30

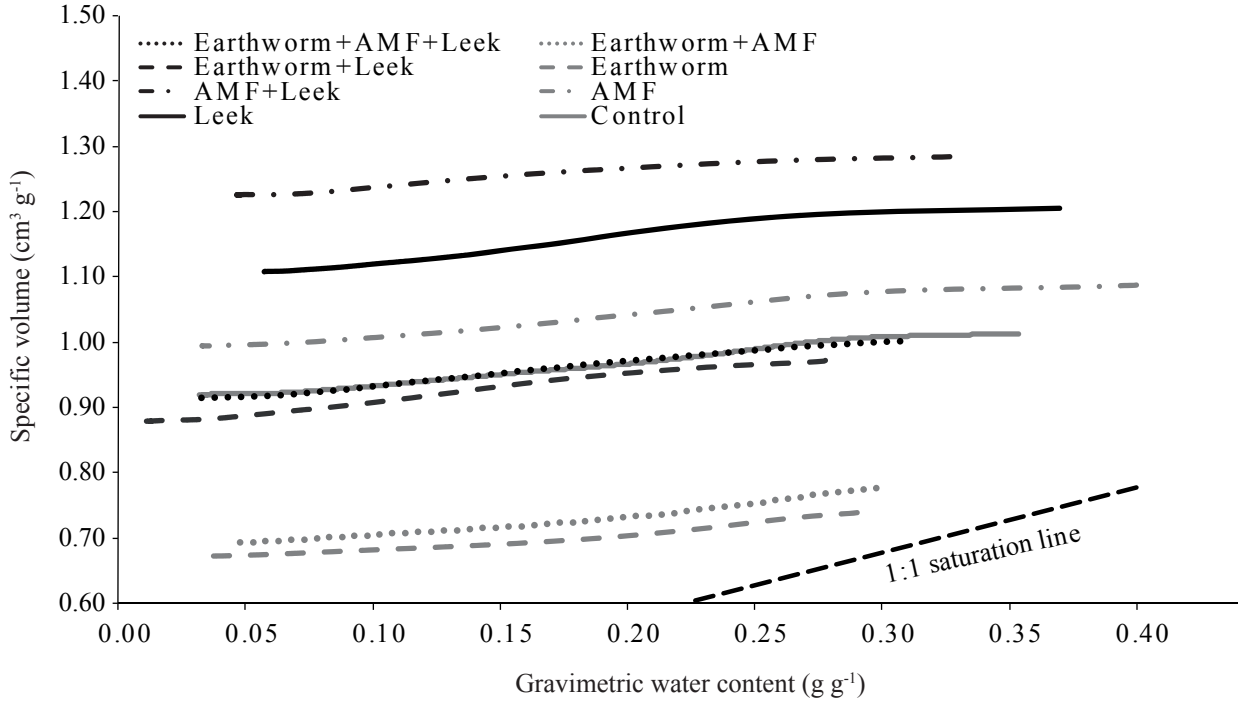

Fig. 4.2 Shrinkage curves of the eight treatments representing all the combinations of the presence/absence of the three factors: Leek roots (*Allium porrum*), Earthworms (*Allolobophora chlorotica*) and AMF (*Glomus intraradices*) with saturation 1:1 line (large dashed line). According to the experimental design, no confidence intervals can be given.

Table 4.2 Swelling capacity of the soil (SC) and the plasma (SC_p), slope of the structural shrinkage (K_{Str}), of the basic shrinkage (K_{Bs}), water content (W), soil specific volume (V) at the transitions points SL, AE, ML and MS, available water (AW), easily available water (EAW), and corresponding plasma- (Vp) and structural (Vst) porosities as determined with fitting the XP model on the shrinkage curves established for each microcosm. E: Earthworm, A: AMF, L: Leek roots, C: control. Water contents are determined with a 10^{-4} g g^{-1} resolution, soil and structural pore volumes with a 0.01 cm^3 g^{-1} resolution, and plasma pore volumes with a 10^{-4} cm^3 g^{-1} resolution.

	Treatment							
Bulk soil properties	E+L	E	A+L	A	E+A+L	E+A	L	C
SC (%)	10.0	7.3	4.5	7.6	8.8	8.8	8.6	9.9
K_{Str} (cm^3 g^{-1})	0.178	0.279	0.105	0.075	0.258	0.309	0.023	0.061
K_{Bs} (cm^3 g^{-1})	0.462	0.467	0.365	0.393	0.395	0.555	0.538	0.380
W_{SL} (g g^{-1})	0.021	0.141	0.056	0.091	0.049	0.134	0.063	0.046
V_{SL} (cm^3 g^{-1})	0.879	0.688	1.226	1.004	0.916	0.712	1.108	0.920
W_{AE} (g g^{-1})	0.053	0.232	0.081	0.140	0.072	0.262	0.151	0.106
V_{AE} (cm^3 g^{-1})	0.888	0.715	1.230	1.018	0.921	0.759	1.140	0.934
W_{ML} (g g^{-1})	0.168	0.254	0.120	0.266	0.176	0.276	0.210	0.292
V_{ML} (cm^3 g^{-1})	0.941	0.725	1.245	1.068	0.962	0.767	1.172	1.005
W_{MS} (g g^{-1})	0.259	0.289	0.292	0.328	0.285	0.295	0.339	0.332
V_{MS} (cm^3 g^{-1})	0.967	0.738	1.281	1.080	0.997	0.774	1.203	1.011
AW (g g^{-1})	0.205	0.058	0.211	0.188	0.214	0.032	0.188	0.226
EAW (g g^{-1})	0.090	0.035	0.172	0.062	0.109	0.019	0.130	0.040
Calculated plasma Vp and structural porosity Vst								
Vp (SL) (cm^3 g^{-1})	0.040	0.194	0.070	0.119	0.062	0.209	0.114	0.081
Vst (SL) (cm^3 g^{-1})	0.462	0.117	0.779	0.507	0.477	0.126	0.616	0.462
Vp (AE) (cm^3 g^{-1})	0.053	0.232	0.081	0.140	0.072	0.262	0.151	0.106
Vst (AE) (cm^3 g^{-1})	0.457	0.106	0.772	0.501	0.472	0.119	0.612	0.451
Vp (ML) (cm^3 g^{-1})	0.168	0.254	0.120	0.266	0.176	0.276	0.210	0.292
Vst (ML) (cm^3 g^{-1})	0.395	0.094	0.747	0.424	0.409	0.113	0.584	0.336
Vp (MS) (cm^3 g^{-1})	0.206	0.269	0.192	0.292	0.222	0.284	0.264	0.308
Vst (MS) (cm^3 g^{-1})	0.384	0.091	0.712	0.411	0.398	0.113	0.561	0.325
SCp (%)	415.35	38.93	173.16	144.21	256.47	36.13	130.78	281.53

Shrinkage analysis

The bulk soil shrinkage curves and the parameters of the fitted XP model are presented in Fig. 4.2 and Table 4.2, respectively. According to the precision of the transducers we used, the water contents were determined with a 10^{-4} g g^{-1} resolution, soil and structural pore volumes with a 0.01 cm^3 g^{-1} resolution, and plasma pore volumes with a 10^{-4} cm^3 g^{-1} resolution. Moreover, the coefficients of variation of the shrinkage parameters as determined on neighboring soil samples collected in a field were estimated as below 10 % (water contents) and 3 % (volumes) by Boivin (2007), thus giving an overestimation of the standard errors associated with our measurements performed on homogenized soils. The changes commented below are larger than the corresponding errors (Table 4.2).

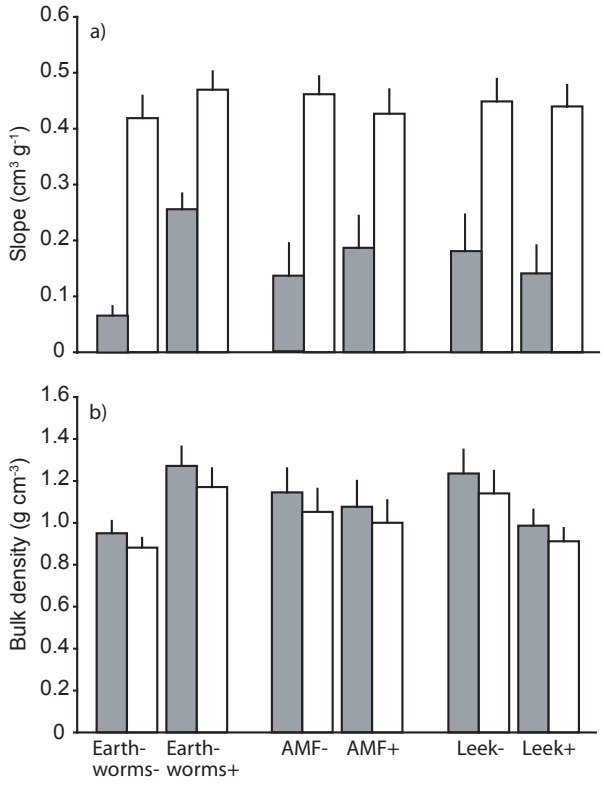

Fig. 4.3 Isolated effect of the three factors (earthworms, *Allolobophora chlorotica;* AMF*, Glomus intraradices* and Leek roots, *Allium porrum*), as illustrated by calculated average values of a) the slope of the structural shrinkage (grey) and the slope of the basic shrinkage (white) and b) the bulk soil density at SL (grey) and MS (white), with or without the factor in the corresponding treatment. Bar represents mean + SE as calculated from ANOVA.

The samples with the AMF, Leek or AMF + Leek treatments presented a larger specific volume than the control. On the contrary, Earthworm treatment resulted in smaller specific volume of the soil than the control. Interestingly, the shrinkage curves of the control and the treatment containing Earthworm + AMF + Leek roots had a similar specific volume and water content range (Fig. 4.2).

We observed a significant effect of Earthworm on the mean slope of the structural shrinkage (K_{str}, $P < 0.05$) (Table 4.2). The mean slope of the structural shrinkage was 0.26 (*SE* = 0.03) with earthworms whereas the slope was 0.07 (*SE* = 0.02) without earthworm (Fig. 4.3a). The specific bulk volume (*V*) at SL and MS was significantly decreased by Earthworm ($P < 0.05$) and increased by Leek roots ($P < 0.05$) (Table 4.2). The bulk soil density (ρ), calculated as the inverse of the specific bulk volume, was therefore increased by earthworms at SL and MS and decreased by leek roots (Fig. 4.3b). The structural porosity was increased by Leek roots, AMF, and their combined effect and decreased by Earthworm at SL, AE, ML and MS points (Table 4.2). Accordingly, Earthworm and Earthworm + AMF treatments reduced the plant available water (AW) and easily available water (EAW) (Table 4.2). The compacting effect of earthworms was not mitigated by AMF, was partly mitigated by leek, and almost fully mitigated by AMF + Leek. Interestingly, the increase in structural volume observed with the Leek + AMF treatment was larger than the addition of the structural volume increase observed with the single Leek and AMF treatment, thus revealing a synergistic effect, particularly at lower water content.

Plasma pores

All the plasma volumes are close to the control except the treatments Earthworm and Earthworm + AMF (Fig. 4.4). In these cases, the plasma air entry values occurred at larger soil water content than with the other treatments. Moreover, Table 4.2 shows that $V_{p,SL}$ is more than two times larger for the Earthworm and Earthworm + AMF treatments compared to the other treatments, leading to a smaller swelling capacity of the plasma (SCp). The observed differences in volumes, however, are small compared to the volume changes observed on the structural pores with the different treatments.

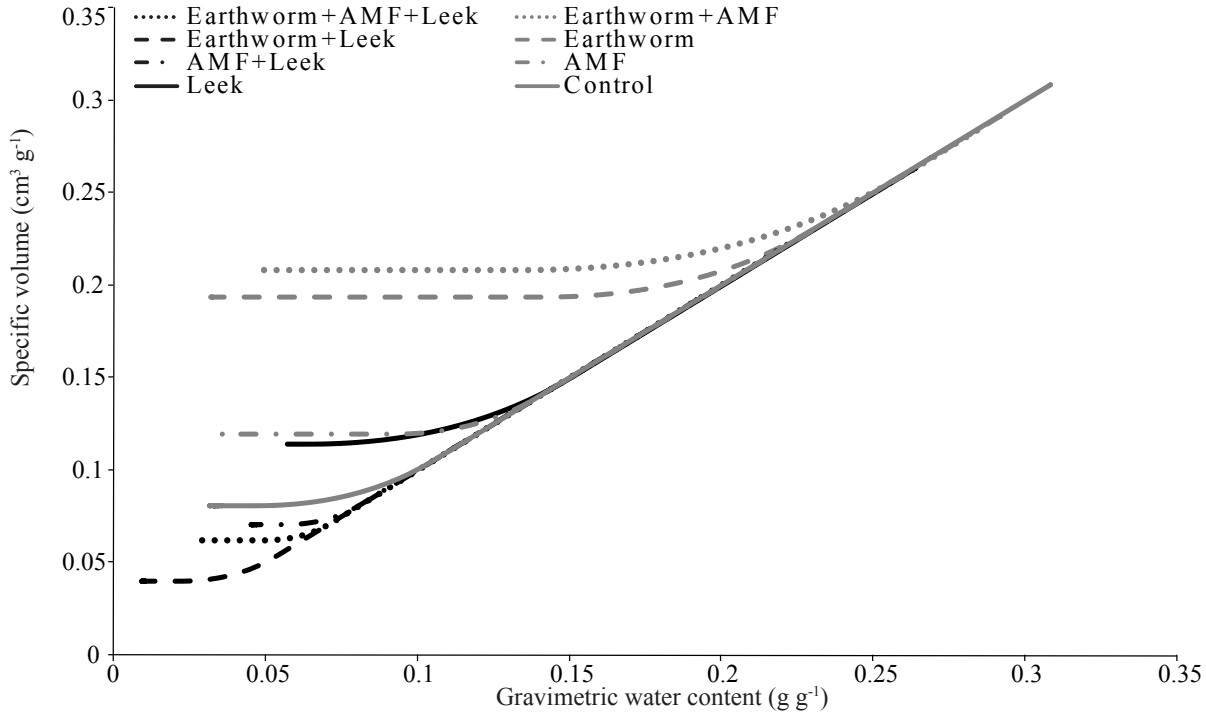

Fig. 4.4 Plasma shrinkage curves from the samples of the eight treatments representing all the combinations of the presence/absence of the three factors: leek roots (*Allium porrum*), earthworms (*Allolobophora chlorotica*) and AMF (*Glomus intraradices*). Specific volumes and gravimetric water contents are expressed in cm^3 and g per gram of soil, respectively. According to the experimental design, no confidence intervals can be given.

Structural pore size distribution

Fig. 4.5 presents the cumulated volume of the structural pore size distribution, corresponding to the water saturated structural pores. The structural pore volumes of the Earthworm and the Earthworm + AMF treatments were similar (Fig. 4.5a), smaller at every pore size than the control, and the smallest radius of structural pores were larger with Earthworm than with the control. The smaller structural pore radius was 11 µm for the control, 16 µm for the Earthworm treatment and 25 µm for the Earthworm + AMF treatment. Only the Earthworm + AMF + leek roots treatment had a structural pore volume larger at every pore size than the control, and the smallest structural pore size were 3 µm radius. The cumulated pore size distributions of the different treatments including leek roots are presented in Fig 4.5b. The structural pore volumes with plant roots were higher than the control at any pore size, and the smallest structural pore radii were always smaller than the control. Fig. 4.5c shows the structural pore size distribution of the treatments containing AMF. As previously described in Fig. 4.5a, the curve of the AMF + Earthworm treatment was below the control curve. The three other treatment curves showed larger volumes than the control. The volumes were larger with the AMF and the AMF + Leek treatments than with the Earthworm + AMF + Leek. Unlike leek roots, AMF alone did not generated smaller structural pores than the control.

The results above are enforced by the values of the air filled structural pore volumes at water saturation (-10hPa), corresponding to the coarser (larger than 150 µm) structural pore volume (Table 4.1). The leek + AMF, AMF,

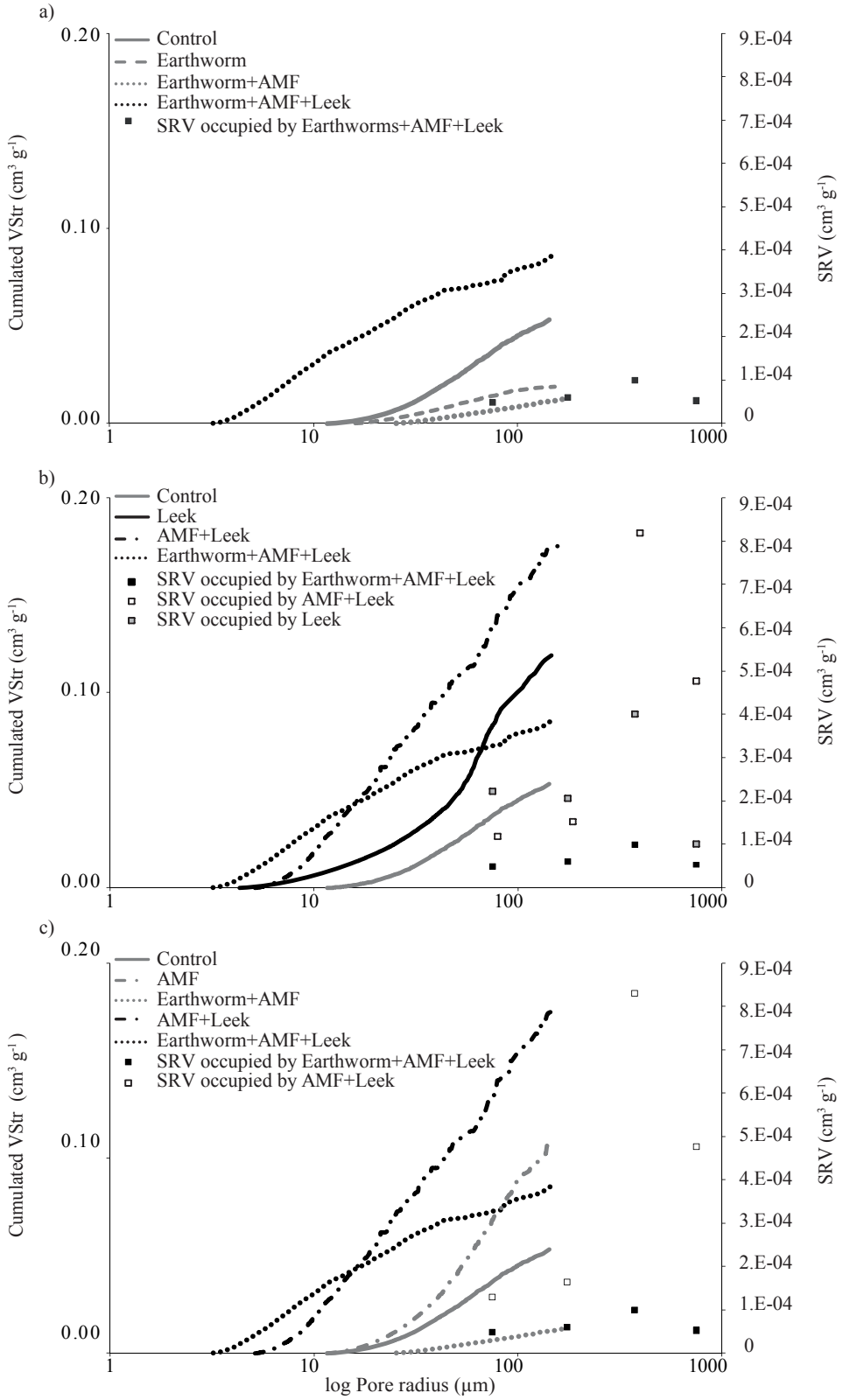

Fig. 4.5 Cumulated structural pore volume vs. equivalent pore diameter for a) Earthworm treatments, b) Leek roots treatments and c) AMF treatments. Pore volumes are calculated with the Jurin-Laplace law for pores smaller than 150 µm (-10hPa). Air filled pore volumes at -10hPa are presented in Table 4.1. Squares represent the specific root volume (SRV) per class of root diameter. Data of the Earthworm + Leek treatment not available.

and Leek treatments showed a greater air filled structural pore volume than the control and a smaller air filled pore volume for Earthworm and Earthworm + AMF treatments. Compared with the air filled structural pore volumes, the root or AMF volumes in the soil was about 3 orders of magnitude smaller.

Discussion

Shrinkage analysis revealed a different impact of the investigated soil biota on the soil physical properties.

At any water content, the presence of earthworms resulted in a decrease of the specific bulk volume and in a significant increase in the bulk soil density. The physical changes induced by *Allolobophora chlorotica* included a decrease of the structural pore volumes at any pore size, a disappearing of the smallest structural pore radii, a decrease in plant available water, and a hardening of the plasma. Thus, we demonstrated that *Allolobophora chlorotica* compacted the soil. Soil compaction by earthworms was described by some authors with tropical endogeic earthworms (see Blanchart et al. 2004 for a review). In particular, the authors suggest that in Amazonia the compaction effect was induced by rapid changes in land use and mainly due to the proliferation of an endogeic species called *Pontoscolex corethrurus* during the reconversion of forests to pasture (Chauvel et al. 1999). To some extent, our experiment produces similar conditions, as the only earthworm species introduced was an endogeic species. Blanchart et al. (1997) described two functional groups within endogeic earthworms: compacting and decompacting species. They suggest that the presence of both types of earthworms is necessary to maintain the natural soil structure. If one or both types of earthworm are excluded from the soil, the initial structure is greatly affected.

We also observed an increase of the slope of the structural shrinkage (K_{str}) with *Allolobophora chlorotica*, thus indicating a decrease in the hydro-structural stability of the soil upon drainage of the structural pores (Schaffer et al. 2008) in the presence of earthworms. This is in agreement with the findings of Milleret et al. (2009) based on six replicated measurements of the structural stability with the wet-sieving method on the same experiment. The percentage of water stable macro aggregates (i.e aggregates > 250 μm measured in the 1-2 mm size class) was significantly decreased with earthworms. To our best knowledge it is the first time that wet sieving aggregate stability and hydro-structural stability are measured on the same samples. The good agreement between the two methods seems promising and suggests further comparison.

Finally, the changes observed on plasma swelling may be attributed to more rigid particles (Tessier 1980; Tessier et al. 1992) that is a hardening of the plasma by earthworms, which was not observed with Earthworm combined with Leek root treatments.

Although soil compaction attributed to endogeic earthworms was already reported (see above), our findings are largely in contradiction with the current knowledge of earthworm impacts on soil physical properties. Many studies highlighted a positive effect of earthworms on soil structure, soil aggregation or soil water infiltration (Edwards and Bohlen 1996). In particular it has been demonstrated that earthworms enhance soil stability, especially when casts are ageing and drying (Shipitalo and Protz 1989). We can comment on this apparent discrepancy as follows. First,

most studies focused on anecic species and compared surface casts with the bulk soil. It is likely that our results apply specifically to some endogeic species. The casts of *Allolobophora chlorotica* are not deposited at soil surface, thus limiting ageing of the casts upon drying cycles. Second, in natural conditions earthworms population is a mix of all ecological categories (i.e. anecic, epigeic and endogeic), and soil physical properties result therefore from a complex equilibrium. The effects of functionally different earthworm species on soil aggregation have been studied and the results highlighted that different earthworm species differently affected the incorporation of fresh organic matter and soil stability, and that interactive effects between different earthworm species must be considered (Bossuyt et al. 2006). In our experiment, one species only was used. Third, the earthworm density we used was high for endogeic species alone compared with field observed earthworm density. We applied this density to emphasize the effect of the selected earthworm, as applying a field relevant density for *Allolobophora chlorotica* alone would have led to negligible effect at microcosm scale. Our results draw, therefore, the attention to the possible effect of one single species proliferating, as described in a particular field case by Blanchart et al. (2004). This also underlines the interest for further research on earthworm species interactions. The general case of multi-earthworm species in microcosm experimental conditions using shrinkage analysis in temperate soils remains, therefore, to be experimented.

On the contrary, leek roots decreased the bulk soil density despite an initial bulk density of 1.15 g cm^{-3}. This increase in volume was accompanied with a enhanced hydro-structural stability, a larger structural pore volume at any pore size, smaller structural pore radii and an increase in plant available water. The generated structural pore diameters were smaller and larger than the roots, volume of which was much smaller than the generated pore volumes. Leek root diameters were mostly in the range of pore diameter greater than 150 µm (Fig. 4.5), which corresponds to air-filled pores at -10 hPa. This result is in accordance with O'Keefe and Sylvia (1992) who showed that AMF and root hairs were of diameter that would allow them to penetrate pores that hold water at water contents less than field capacity while root would be excluded from these pores. The new structural pores were, therefore, not generated by the mechanical effect of root growth, but most likely by the induced microbial activity and the resulting self-organisation of the soil-microbe complex (Feeney et al. 2006; Young and Crawford 2004).

Regarding AMF, they induced a decrease in soil bulk density and structural pore volume analogous to that of roots though less pronounced. However, AMF alone did not develop small-diameter structural pores. The size and volume of the generated structural pores were larger than the size and volume of the AMF, suggesting an indirect effect of the mycorrhizae.

The combined treatment revealed different interactions. Obviously, AMF could not mitigate the compaction induced by *Allolobophora chlorotica*, Leek roots partly mitigated the effect of the earthworm, and it is only AMF + leek root that allowed keeping soil physical properties close to the control. The effects of roots were identified as identical in all the treatments, in particular the generation of very small diameter structural pores. The structure generation due to AMF and roots revealed a positive synergistic effect at lower water content in agreement with

the stimulation of plant growth by AMF. This is in accordance with studies demonstrating that the presence of plant have the greatest impact on structure generation, with AMF also contributing to accentuate soil stability (Hallett et al. 2009; Jastrow et al. 1998). The possibility of differential AMF response to soil compaction was described by Nadian et al. (1998). As the diameter of external hyphae is smaller than roots it may penetrate smaller pores and enhance the observed leek roots effect and stimulate plant exudates secretion, thus increasing bacterial activity.

Conclusions

In the present study, shrinkage analysis was successfully applied to the assessment of the physical impact of soil biota in soil microcosms. To our best knowledge, such application of shrinkage analysis was not reported previously. Although performed on a limited number of samples, the provided results seem promising for this kind of investigation. The advantages of the method are both the accuracy of the determination, thus revealing small changes, and the large spectra of properties determined, thus allowing a full description of small concomitant changes.

A compacting and destabilizing effect of *Allolobophora chlorotica*, and a de-compacting and stabilizing effect of AMF and leek roots were revealed. Interestingly, a synergistic effect of roots and AMF in the absence of the earthworm was also highlighted, and this synergistic effect was not observed in presence of the earthworm. Combining ShC and WRC analysis allowed comparing the structural pore size distribution in the sampled treatments. This analysis showed that the structural pore volume generated by root and AMF growth was several orders of magnitude larger than the volume of the organisms and that the new structural pore diameters were not the same as those of the organisms. We, therefore, show that these pores were not generated by mechanical intrusion of the biota in the soil. More likely, root exudates as well as other AMF secretion serve as carbon source for microorganisms that in turn enhance soil aggregation and porosity. These changes resulted in more porous and stable soils with larger plant available water induced by AMF and plant roots. Our results, therefore, support the idea of a self-organization of the soil-plant-microbe complex as previously suggested by Young and Crawford (2004).

Acknowledgments

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5

Impact of two root networks, earthworms and mycorrhizae on soil physical properties and plant production of an unstable silt loam Luvisol

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In preparation

Abstract

Soil organisms are known to play a crucial role on soil ecological processes as organic matter turnover, nutrient cycling and engineering of the soil physical properties. They are essential for soil fertility and plant performance. However, their role on the numerous soil physical properties is still scarcely studied. Among the techniques used for studying the soil physical properties, shrinkage analysis was successfully applied in a preliminary microcosm study to assess the physical impact of soil biota. The objectives of the present study were therefore to test the effect of two plants (leek, *Allium porrum* and petunia, *Petunia hybrida*), mycorrhizae (*Glomus intraradices*) and earthworms (*Allolobophora chlorotica*) on soil physical properties. This 22-week microcosm study was performed under glasshouse and used an unstable silt loam Luvisol. Leek and petunia differently affected soil physical properties. The specific bulk soil volume and the pore volumes at any pore size was increased with leek compared with petunia or unplanted microcosms. In comparison with unplanted microcosms, the presence of plant increased the soil structural stability and this stability was greater with petunia than leek roots. The root architecture of both species may explain the results.The leek root architecture (greater mean root diameter, less branched) increased the specific soil volume and porosity, while the petunia root network increased soil stability with a better physical root enmeshment of soil. Despite a low mycorrhization rate, mycorrhizae increased the percentage of water-stable macroaggregates and the specific bulk soil volume. The presence of earthworm without plants decreased the specific bulk volume of the soil and the pore volumes at any pore size. This effect was however reduced when plants were added in the microcosm. In conclusion, shrinkage analyses confirmed that soil organisms are able to modify soil physical parameters and that root architecture is an important factor controlling soil stability. Our results therefore support the idea that the soil response is varying both in function of soil organisms and soil type.

Keywords

Shrinkage analysis (ShC), soil porosity, earthworms, arbuscular mycorrhizal fungi (AMF), plant root network, soil structure, structural stability.

Introduction

Soil organisms are known to play a crucial role on soil ecological processes as organic matter turnover, nutrient cycling and engineering of the soil physical properties. They are therefore essential for soil fertility and nutrient uptake by plants (Bradford et al. 2002; Wardle et al. 2004). The soil physical habitat is widely assumed to be of prime importance in determining and regulating biological activities (Young and Crawford 2004). As a result, interactions between soil physics and the biological and chemical processes are key determinants of ecosystem health (Feeney et al. 2006), but are still largely to be deciphered.

Plant roots, microorganisms (bacteria and fungal mycelium) and soil fauna as earthworms or termites are considered to be major factors controlling soil aggregation and porosity (Amezketa 1999; Jastrow and Miller 1991; Six et al. 2004; Tisdall and Oades 1982). As reviewed in Angers and Caron (1998), roots affect soil structure through direct and indirect mechanisms as root penetration creating porosity and favouring water transport, or soil enmeshment and root exudation increasing soil aggregation and stability or enhancing microbial activity, which in turn will affect soil structure. Arbuscular mycorrhizal fungi (AMF) are also known to influence soil physical processes, but the separation of the effects of roots versus AMF (at the level of soil particles entanglement and glue-substance secretion as glomalin) is still under debate due to the symbiotic relationship between plants and AMF (Hallett et al. 2009; Jastrow et al. 1998; Thomas et al. 1993). Through burrowing, casting and mixing of litter and soil (bioturbation), earthworms influence structure, stability, water infiltration and aeration of soils (Edwards and Bohlen 1996). Furthermore plant roots, AMF and earthworms do not act individually but many interactions occur in the soil. Recent studies showed that individual effects of soil organism groups may cancel out each other in combination (Bradford et al. 2002; Wurst et al. 2008). Precise and accurate measurements are subsequently very important for better understandings of these interactions, especially when studying their effects on a very heterogeneous media as the soil with numerous and complex properties.

Among the techniques used for studying the numerous soil physical properties (e.g. soil density, soil structural stability, pore and aggregate size distribution, hydraulic conductivity), shrinkage analysis has been shown to be effective. The soil shrinkage is commonly defined as the soil specific volume change with water content (Haines 1923). Soil shrinkage analysis therefore allows determining many soil properties in a single experiment, like i) the volume, air and water content of plasma and structural pores at any soil water content (Braudeau et al. 1999; Braudeau et al. 2004), ii) the soil hydro-structural stability (Schaffer et al. 2008) and iii) water retention curves (WRC) (Boivin et al. 2006). Shrinkage analysis is based on the simultaneous and quasi continuous measurement of shrinkage curve (ShC) and WRC (Boivin et al. 2006) and the modelling of the ShC with the XP (eXPponential) model (Braudeau et al. 1999) or the equivalent PS (PedoStructure) model (Braudeau et al. 2004, see Milleret et al. (2009) for more details on the theoretical description of shrinkage analysis).

In a previous preliminary microcosm study (Milleret et al. 2009), shrinkage analysis has been successfully applied to assess the physical impact of soil biota in a microcosm experiment. The soil used was a carbonated loamy Anthrosol with a well developed and

stable structure. While performed on a limited number of samples, results of the shrinkage analysis showed that, at any water content, endogeic earthworms decreased the specific bulk volume and hydro-structural stability and increased bulk soil density. On the contrary, leek roots decreased the bulk soil density and increased the hydro-structural stability. Moreover, a positive synergistic effect between AMF and roots in the absence of earthworms was highlighted.

Following the promising results of the preliminary experiment, we performed a new microcosm experiment with replication of treatments in order to study the effects of the same earthworm, AMF and plant species on soil physical parameters. Furthermore, we choose to employ a soil having different physico-chemical properties in order to test whether the effects of these soil organisms vary with the type of soil used. For this purpose, the first 30 cm of a silt loam Luvisol characterized as unstable and sensitive to crusting according to Le Bissonnais (1996) was used. In particular we tried to assess if: i) soil organisms as AMF (*Glomus intraradices*, Schenk & Smith), endogeic earthworms (*Allolobophora chlorotica*, Savigny) and plant roots were able to affect the physical properties of this soil ii) two different root networks, i.e. the leek (*Allium porrum*), already used in the previous experiment, and the petunia (*Petunia hybrida*) differently influence the soil physical properties. In addition to shrinkage analysis, the percentage of water-stable macro-aggregates (i.e. > 250 µm) and biological interactions between the soil organisms were investigated.

Based on our previous results (Milleret et al. 2009) we hypothesize that earthworms would increase the bulk soil density and the hydro-structural stability. In parallel, we suppose that the addition of plants would show opposite results. According to their different root architecture, leek and petunia are hypothesized to differentially influence soil physical properties. The petunia root system is supposed to be thinner and more branched than the leek, thus suggesting increased soil stability in the presence of petunia compared with leek roots.

Material and methods

Experimental setup, plant, mycorrhiza and earthworm

The soil used in this experiment was a silt loam Luvisol sampled at the experimental site of the Institut National de la Recherche Agronomique – INRA – (48°48'29"N, 2°04'58"E), at Versailles city, France (Balabane 2005; Consentino 2006). The texture was of 167 g kg^{-1} clay, 562 g kg^{-1} silt and 271 g kg^{-1} sand, with a total carbon content of 9.0 g kg^{-1}, Ct/Nt: 9.3, pH (H_2O) of 7.0. It had been cultivated for more than 50 years with conventional tillage (mouldboard plow at 0-30cm) with a rotation based on wheat (*Triticum aestivum* L.), colza (*Brassica napus* L.) and pea (*Pisum sativum* L.).

The soil was carefully sampled from one "buffer" band of the parcel of the Dmostra project (Balabane 2005) at 0-25 cm depth (Ap horizon) with shovels to keep the natural structure of the soil as much as possible.

The soil was air-dried, sieved to 2 mm size aggregates, homogenized and sterilized by autoclaving (one hour at 121°C for two consecutive days) prior to repacking in the microcosms (PVC tube; 35 cm height and 15 cm internal diameter) with a bulk density of 1.18 g cm^{-3}. Afterwards, a 65 ml soil suspension (100 g of soil dispersed in 1000 ml of autoclaved

distilled H_2O and filtered on 11 µm paper) was added to re-inoculate the sterilized soil with microorganisms, but without AMF (Koide and Li 1989).

Treatments were applied depending on the possible combinations of the three following factors: 1) the three levels of plant species, i.e. unplanted, leek (*Allium porrum* var. Mercure) or petunia (*Petunia hybrida* W115), 2) the presence/absence of AMF (*Glomus intraradices*; 30 g of spores and hyphae) and 3) the presence/absence of endogeic earthworms (*Allolobophora chlorotica*; five individuals of equal biomass (0.66 g ± 0.01 g). Treatments containing AMF without plants are not possible to produce due to the obligate symbiosis between plant roots and AMF. Consequently, according to the presence or absence of earthworm (E), leek (L), petunia (P) and AMF (A), a total of ten treatments were applied (C, E, L, P, L+E, P+E, L+A, P+A, L+E+A, P+E+A). The control C corresponds to the treatment without plant, earthworm and AMF. All treatments were replicated three times resulting in a total of 30 microcosms. We selected an endogeic species because this kind of earthworms inhabit the organo-mineral soil horizon feeding on the soil organic matter closely linked to the mineral matrix; as a matter of fact, they consume more soil than other ecological categories to fulfil their nutritional requirements and consequently largely burrow within the upper centimetres of the soil (Capowiez 2000; Lee and Foster 1991).

The microcosms were kept 22 weeks in an experimental greenhouse in Jussy (Geneva, Switzerland) under the following conditions: photoperiod 16/8 h (day/night), temperature 16 ± 2 °C, 50% moisture content. Irrigation was performed twice a week using a modified Hoagland's nutrient solution without P in order to promote the AMF-plant symbiosis. Every three weeks, each microcosm was weighted and adjusted to equal soil water content with deionised water.

Harvesting and sampling

After 22 weeks, leek shoots were cut at ground level, pooled, air-dried and weighed. Four undisturbed soil cores of approximately 100 cm^3 were removed from the middle of the microcosms for soil shrinkage curve (ShC) and water retention curve (WRC) measurements. The remaining soil was thoroughly mixed and roots were sampled in a fraction of 500 g of soil. The roots were therefore carefully washed, air-dried and weighted. Earthworms were hand-collected, counted and weighed.

Mycorrhiza analysis

To measure AMF root infection, roots were first cleared in 10% KOH, acidified in 1% HCl and stained in 0.05% Trypan blue in lactoglycerol. The AMF colonisation was determined on 150 root segments at 250x magnification using a modified line intersect method (McGonigle et al. 1990).

Soil analysis

Macroaggregate water-stability

The water-stable soil macroaggregates in the 1-2 mm size class ($WSA_{1\text{-}2mm}$) were determined using the wet-sieving apparatus (Kemper and Rosenau 1986). A 250 µm sieve was filled with a 4 g sample of 1-2 mm air-dried aggregates. The samples were then moistened by capillarity with deionised water for 10 minutes and wet-sieved 10 minutes more with a stroke length of

19 min^{-1}. The WSA corresponded to the amount of macroaggregates (> 250 µm) remaining on the sieve and was expressed as a percentage of the total initial mass of soil after correction for the weight of coarse particles (> 0.25 mm).

Shrinkage and retention curve analysis

Quasi-continuous shrinkage curves (ShC) and water retention curves (WRC) were determined on undisturbed sub samples of approximately 100 cm^3. The equipment and methods used are the same as presented in Boivin et al. (2004) and Milleret et al. (2009). We wetted the soil samples with deionised water by applying a water potential of –10 hPa with respect to the centre of the samples. This means that pore radius up to 150 µm were filled with water.

During drying, the samples were placed on electronic balances (0.01 g precision) contained in a thermostatic chamber at 20°C. Calibrated displacement transducers (resolution of 1 µm) were used to measure changes in sample height during drying. Tensiometers (ceramic cups; length 2.0 cm, diameter 0.2 cm) connected to pressure transducers were inserted in the middles of the samples to measure the water potential. Weight, height and water potential were recorded at intervals of 5 minutes until the sample weights reached constant values, which took about 4 days. Then, the dry sample volumes were determined by means of hydrostatic weighing with the plastic bag method described by Boivin et al. (1990), and the samples were dried in an oven at 105 °C for 24 hours to obtain the dry weight.

Changes in sample height were converted to changes in specific bulk sample volume by

$$V = V_E \times \left(\frac{H}{H_E} \right)^3 , (1)$$

where the exponent 3 denotes isotropic shrinkage (e.g. Boivin 2007), V_E and H_E are the specific bulk volume and height at the end of the experiment, and V and H are the bulk volume and height during the experiment.

The XP model equations (Braudeau et al. 1999) were subsequently fitted to the experimental shrinkage data by a non-linear simplex method (Chen and Saleem 1986) to determine the coordinates of the transition points between the shrinkage domains (Figure 5.1), namely shrinkage limit (SL), air entry (AE), the dry point of structural porosity (ML) and the maximum swelling of the plasma (MS).

The slope of the structural domain K_{Str} was calculated as:

$$K_{str} = \frac{[V(ML) - V(MS)][\exp(1) - 1]}{[W(ML) - W(MS)] - K_{Bs}[\exp(1) - 2]}, (2)$$

where V_{ML}, W_{ML}, V_{MS} and W_{MS} are the volume and water content of the soil at MS and ML, respectively.

Using the XP model equations for the plasma porosity given by Braudeau & Bruand (1993), we then calculated the specific plasma porosity, V_p (in cm^3 g^{-1} of soil), and the plasma water content, W_p (in cm^3 g^{-1} of soil). The specific air content of the plasma, A_p, was calculated as

$$A_p = V_p - W_p , (3)$$

The specific structural porosity, V_s, was calculated as

$$V_s = V - V_p - \rho^{-1} , (4)$$

where ρ^{-1} is the specific volume of the solid phase (set to 1/2.65 cm^3 g^{-1}). At SL and AE this volume corresponds to the specific air content of the structural porosity (*A*s). Moreover, we calculated A_{sat} (in cm^3 g^{-1} of soil) that corresponds to the specific air-filled porosity at saturation as

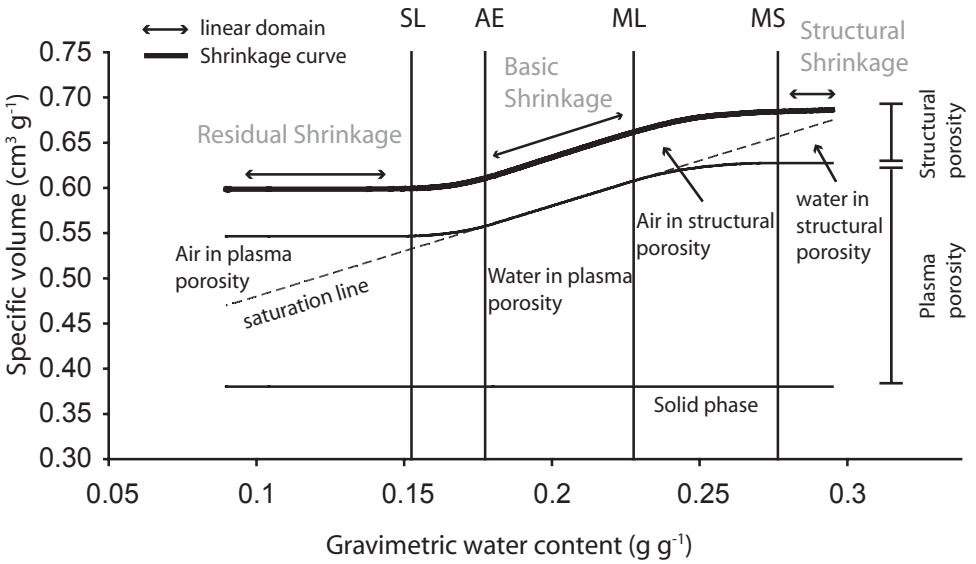

Fig. 5.1 Example of a shrinkage curve with the transition points (SL, shrinkage limit; AE, air entry; ML, macro-porosity limit; MS, maximum swelling), linear domains (residual shrinkage, basic shrinkage, structural shrinkage), and cumulated calculated specific volumes (from bottom to top: solid phase, water in plasma porosity, air in plasma porosity, water in structural porosity, air in structural porosity and bulk soil volume that is shrinkage curve) with saturation 1:1 line.

$$A_{sat} = V - W_{sat} - \rho^{-1}, \quad (5)$$

where W_{sat} (in g g^{-1} of soil) is the total gravimetric water content at saturation. Bulk density was calculated as the inverse of the specific bulk volume, V.

The simultaneous weight and tensiometer measurements were used to determine the water retention curves (WRC). We converted these curves into the pore-size distributions of equivalent cylindrical pores using the Jurin–Laplace equation (e.g. Lawrence 1977).

After ShC and WRC analysis, the undisturbed samples were broken up to measure the root length and volume in each soil sample (see the root size distribution section).

Root size distribution

Specific root volumes in the soil cores were measured as follows. Roots were scanned at high resolution. The dry length, diameter, surface and volume area of the scanned fragments were calculated using an image analysis program (Image J v.1.40, National Institute of Health, USA).

In order to convert the dried root volume (DV) to the fresh root volume (FV), we previously applied the same procedure on fresh leek and petunia root segments that were thereafter dried overnight. This allowed determining the two following regressions:

FV = 1.551 DV + 0.001, (6)

and,

FV = 1.191 DV - 0.0006, (7)

for the leek (r^2 = 0.79, P< 0.001, n = 76) and petunia (r^2 = 0.99, P< 0.001, n = 30) respectively.

The specific root volumes (total and < 250 µm) and the total root length were finally divided by the soil core weight in order to have the total Specific fresh Root Volume (SRV_t), the Specific fresh Root Volume <250 µm ($SRV_{<250}$), and the specific root length (SRL) per gram of soil ($cm^3\ g^{-1}$).

Statistical analysis

We performed the statistical analyses with R 2.6.0 (R Development Core Team 2007). Normal distribution and homogeneity of variance were improved by log-transformation, if necessary, but non transformed means are represented in text and figures (± SE). Analysis of variance (ANOVA) was used to analyze the effects of earthworms (factor with two levels: the presence or absence of *A. chlorotica*) and plant species (factors with two levels: leek or petunia) on root mycorrhization. ANOVA was also used to analyze the effects of AMF (factor with two levels: the presence or absence of *G. intraradices*) and plant species (factors with three levels: unplanted, leek or petunia) on earthworm survival and body fresh weight. In addition, ANOVA was used to analyze the effects of earthworms, AMF and plant species (factors with two levels: leek or petunia) on plant productivity (shoot, root and total biomass and shoot-to-root ratio per microcosm). For the soil analyses, ANOVA was performed to analyze the effects of earthworms, AMF and plant species (factors with three levels: unplanted, leek or petunia) on the percentage of water-stable macroaggregates and the different shrinkage parameters.

Results

Biological activity

The colonization of plant roots in treatments without AMF (control) was negligible (2.9 ± 1.2 %). After 22 weeks, the mean root colonization of the AMF treatments was 20.1 ± 3.4%. Total mycorrhization of plant roots in the AMF treatment was not significantly different between the two plant species ($F_{1,8}$= 1.75, P = 0.22) nor between the presence or absence of earthworms ($F_{1,8}$= 1.21, P = 0.30).

Despite a good activity of earthworms during the experiment, only 11 individuals of the initial 75 were collected after 22-week experiment (14.7 %), which represents only 12.4 ± 3.9% of the initial body fresh weight. However, survival of *Allolobophora chlorotica* was neither affected by plant species ($F_{2,10}$= 0.04, P = 0.96) nor by the presence of AMF ($F_{1,10}$= 0.07, P = 0.80). Similarly, the body fresh weight of earthworms was not affected by plant species ($F_{2,10}$= 0.27, P = 0.77) nor by the presence of AMF ($F_{1,10}$= 0.07, P = 0.80). In every case, visual observation of the soil column after removal of the microcosm indicated that the whole column was burrowed, with burrows reaching the bottom of the column.

Total biomass per microcosm of *Petunia hybrida* (106.5 ± 6.0 g) was greater than *Allium porrum* (22.7 ± 2.5 g; Table 5.1). Shoot biomass of *Petunia hybrida* exceeded that of *Allium porrum* but root biomass did not differ significantly between both plant species (Table 5.1, Fig. 5.2). The presence of AMF increased dried roots (x 3.2) and shoots (x 1.3) of *Petunia hybrida* as well as dried roots (x 2.0) and shoots (x 1.4) of *Allium porrum* (Fig. 5.2). The shoot-to-root ratio was significantly different

Table 5.1 ANOVA table showing the effects of plant species (leek and petunia), earthworms and AMF on total biomass (g), dried roots (g), dried shoots (g), and shoot-to-root ratio. *df* degrees of freedom, MS mean square, **.** P< 0.1, *P<0.05, ** P<0.01, *** P<0.001, ↑ = increase; ↓ = decrease, P>L = petunia greater than leek.

	df	Total biomass			Root biomass			Shoot biomass			Shoot-to-root ratio		
		F	*P*		F	*P*		F	*P*		F	*P*	
Plant species	1	291.01	***	P>L	4.40	.		532.75	***	P>L	151.64	***	P>L
Earthworm	1	0.84			0.41			1.01			0.17		
AMF	1	12.73	**	↑	13.29	**	↑	12.01	**	↑	8.26	*	↓
Plant species x Earthworm	1	0.78			0.19			1.11			0.01		
Plant species x AMF	1	0.62			0.28			0.32			1.79		
Earthworm x AMF	1	0.34			0.06			0.04			0.002		
Plant species x Earthworm x AMF	1	1.83			3.51	.		0.93			3.07	.	
Residuals (MS)	16	0.05			0.20			0.04			0.17		

Table 5.2 Mean values (± SE) of the total Specific Root Volume (SRV_t, $cm^3\ g^{-1}$ of soil), the Specific Root Volume of root diameter < 250 µm ($SRV_{<250}$, $cm^3\ g^{-1}$ of soil) and total Specific Root Length (SRL, $cm\ g^{-1}$ of soil) measured in the soil cores. L: leek, P: petunia, E: Earthworms, A: AMF, *df* degrees of freedom. *P<0.05, **P<0.01, ***P<0.001, † no replicate available.

Treatment		SRV_t	$SRV_{<250}$	Total SRL
L		1.05E-03 (3.55E-04)	4.76E-04 (2.37E-04)	1.45 (0.63)
L+E		9.75E-04 (3.07E-04)	2.82E-04 (1.45E-04)	1.05 (0.29)
L+A		1.25E-03 (1.43E-04)	7.64E-04 (4.34E-05)	2.35 (0.35)
L+A+E		5.15E-04 (9.03E-05)	3.30E-04 (4.38E-05)	1.08 (0.11)
P		1.41E-03 (0.00)†	1.18E-03 (0.00)†	3.25 (0.00) †
P+E		6.62E-04 (5.83E-05)	5.13E-04 (5.94E-05)	3.34 (0.21)
P+A		1.44E-03 (2.91E-05)	1.06E-03 (1.13E-04)	5.08 (1.32)
P+A+E		9.90E-04 (5.95E-05)	7.88E-04 (1.33E-04)	4.92 (0.68)
ANOVA F-value	*df*			
Plant species	1,13	0.29	**11.66 ****	**42.79 *****
Earthworm	1,13	**9.86 ****	**13.50 ****	2.20
AMF	1,13	0.05	2.83	**5.00 ***
Plant species x Earthworm	1,13	0.40	0.28	0.55
Plant species x AMF	1,13	1.41	0.01	1.93
Earthworm x AMF	1,13	0.90	<0.01	0.54
Plant species x Earthworm x AMF	1,13	2.23	2.35	0.12

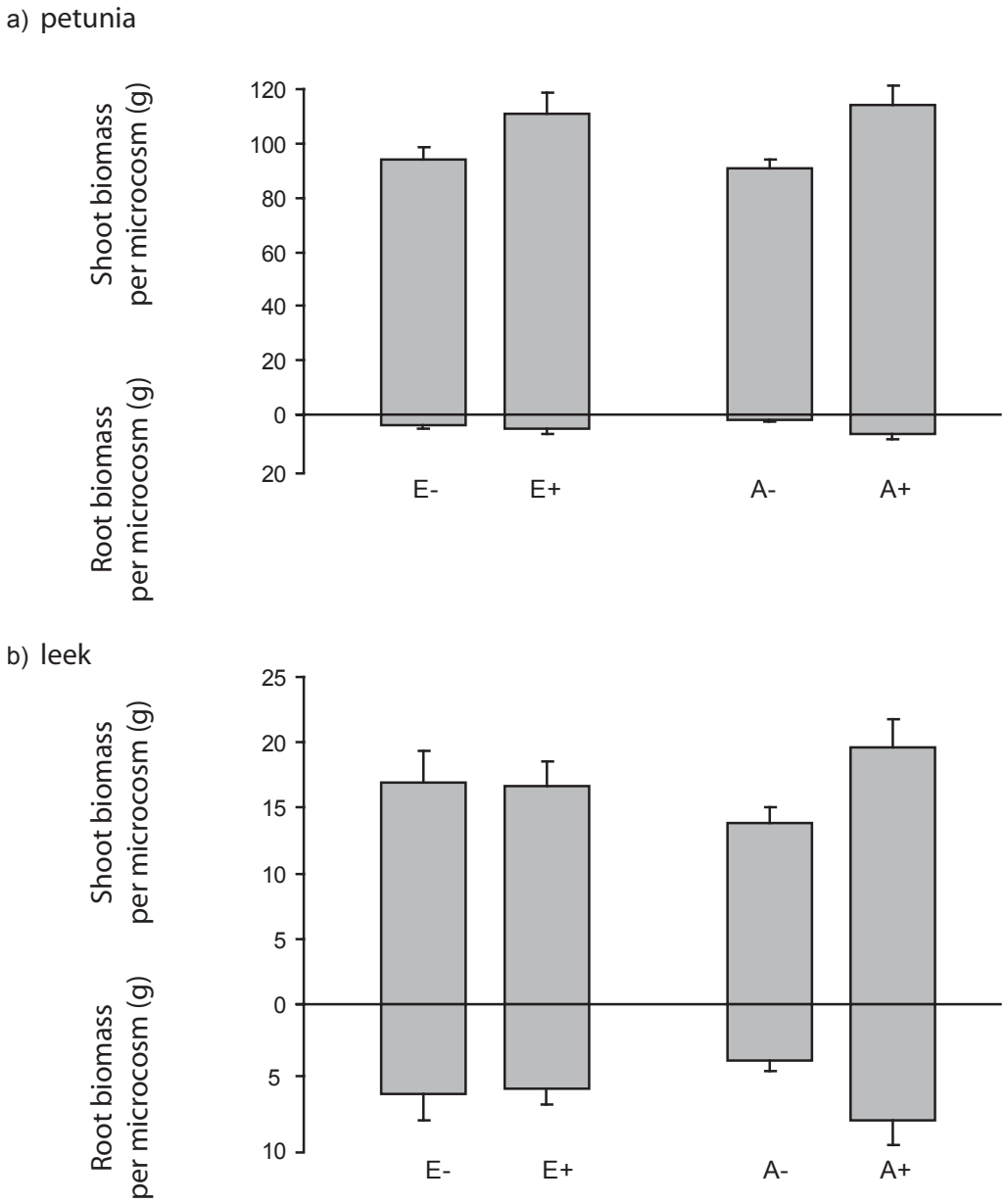

Fig. 5.2 Variation in shoot and root biomass (g dw microcosm^{-1}) of a) petunia (*Petunia hybrida*) and b) leek (*Allium porrum*) as affected by the presence of earthworms (E, *Allolobophora chlorotica*) and AMF (A, *Glomus intraradices*). Bar represents mean + SE.

between plant species and significantly affected by the presence of AMF (Table 5.1). Finally, earthworms did not affect plant productivity in the present experiment and no interaction between earthworms and AMF was measured.

Total Specific Root Volume (SRV_t), Specific Root Volume <250 µm ($SRV_{<250}$) and total Specifi Root Length (SRL) measured in the soil cores used for shrinkage analyses are presented in Table 5.2. While SRV_t was not different between plant species, The $SRV_{<250}$ was greater in the presence of petunia. In both cases, earthworm significantly decreased the SRV. Additionally, the specific length of petunia roots was much greater than leek roots and the presence of AMF significantly increased the SRL of both species.

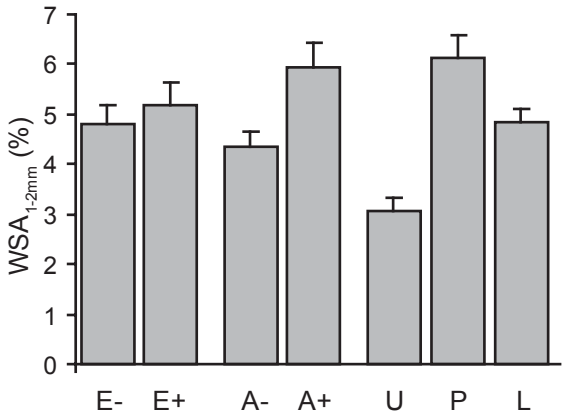

Fig. 5.3 Main effects of the presence of earthworms (E, *A. chlorotica*), AMF (A, *G. intraradices*) and plant species (unplanted, U; petunia, P; leek, L) on the percentage of water-stable macroaggregates in the 1–2 mm size class ($WSA_{1-2\ mm}$). Bar represents mean+SE.

Soil analysis

Macroaggregate water-stability

Overall, the percentage of water-stable macroaggregates in the 1-2 mm size class ($WSA_{1-2\ mm}$) was low, varying between 1.9% and 9.4%. The percentage of $WSA_{1-2\ mm}$ was 1.4 time more stable in the presence of AMF ($F_{1,20}$= 14.620, P = 0.001, Fig. 5.3). In addition, this percentage was significantly affected by the plant species ($F_{2,20}$= 10.31, P < 0.001) in the following manner: petunia > leek > unplanted. In the presence of *Petunia hybrida* the macroaggregates were two times more stable and *Allium porrum* 1.6 times than the unplanted micocosms. The percentage of $WSA_{1-2\ mm}$ was not affected by the presence of earthworm in the microcosms.

Experimental shrinkage curves

Throughout shrinkage analysis, we observed several types of shrinkage curves (Fig. 5.4). In Fig. 5.4a, we observed a S-shape curve as described by Braudeau et al. (1999) with the XP model. In parallel, we also observed S-shape curves with the interpedal phase as described by Braudeau et al. (2004) (Fig. 5.4.b) and doubled S-shape curves (Fig. 5.4.c) that have not been described yet. As shown in Fig. 5.1, shrinkage curves are determined from four transition points named SL, AE, ML and MS. While these points are easily to fit in the first case, the coordinates of ML and MS cannot be strictly identified, especially for the third case (Fig. 5.4c). Further theoretical development

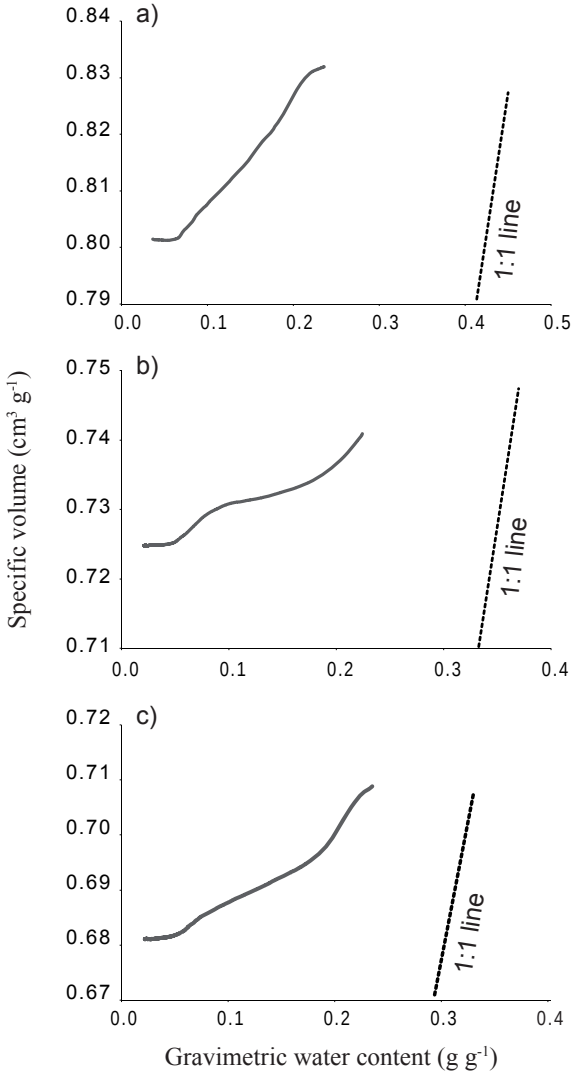

Fig. 5.4 Examples of experimental shrinkage curves obtained in the different soil samples: a) S-shape curve, b) S-shape curve with interpedal phase as described by Braudeau et al. (2004) and c) double S-shape curve.

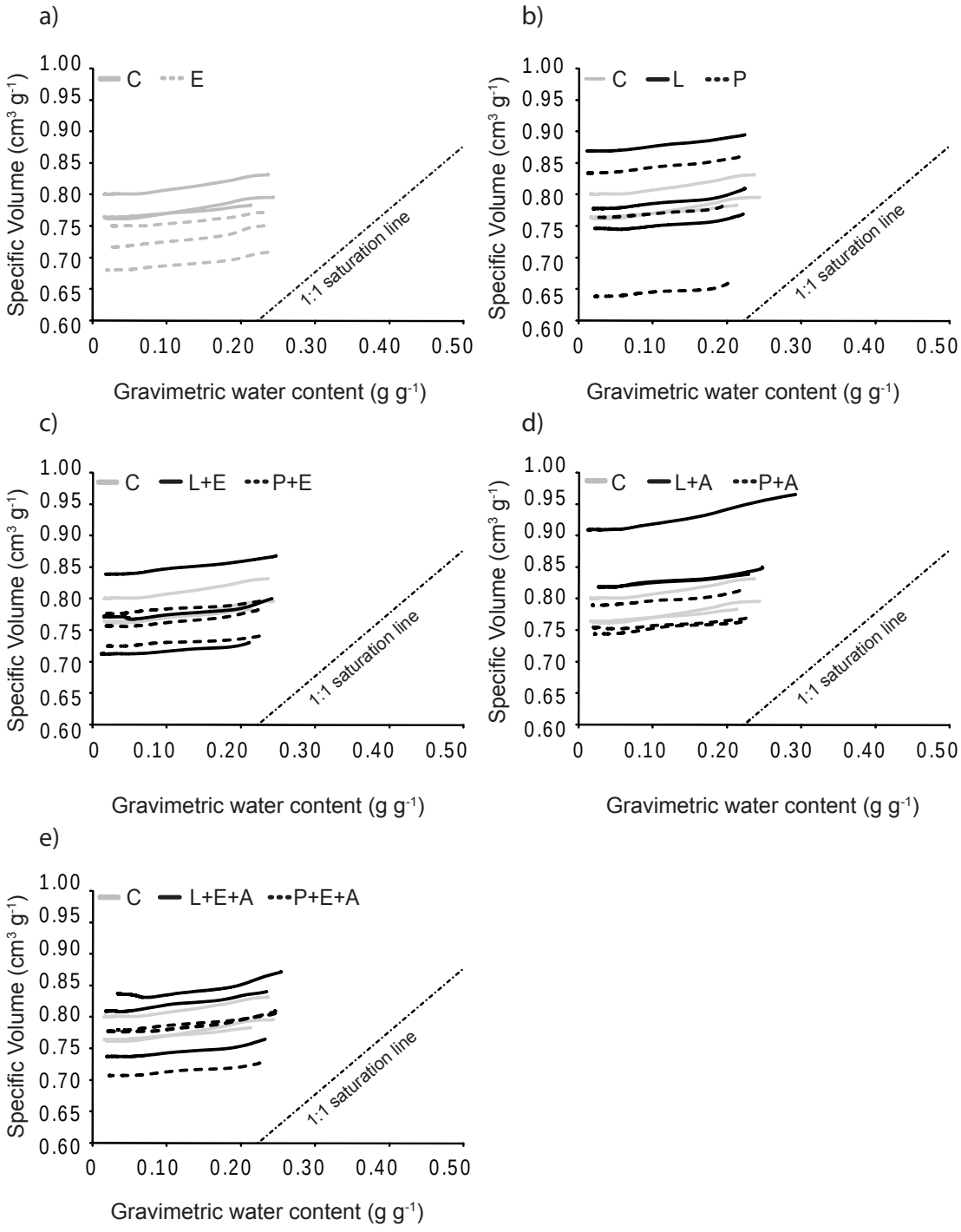

Fig. 5.5 Experimental shrinkage curves of all treatments. Unplanted treatments with earthworm (E, grey dotted lines) in comparison with the unplanted control (C, grey lines) are represented in (a), the plant treatments: leek (L) and petunia (P) in (b), the plant + earthworms treatments (L+E and P+E) in (c), the plant + AMF (A) treatments (L+A and P+A) in (d) and the plant + earthworms + AMF (L+E+A and P+E+A) in (e). For a better understanding of the figure, leek plant treatments, independently of the presence of earthworms and/or AMF, are represented with black lines and petunia plant treatments with black dotted lines. In order to compare the effects of plant species with unplanted microcosms, the control (C) is shown everywhere (b-e).

Table 5.3 Mean values (± SE) of selected shrinkage properties derived from the XP model. Slope of the structural domain (K_{str}, $cm^3\ g^{-1}$), bulk density (ρ ($g\ cm^{-3}$) as the inverse of the specific bulk volume), plasma water content (W_p, $cm^3\ g^{-1}$), specific plasma porosity (V_p, $cm^3\ g^{-1}$), specific air content of the plasma (A_p, $cm^3\ g^{-1}$), specific structural porosity (V_s, $cm^3\ g^{-1}$) at shrinkage limit (SL) and air entry (AE), and air filled porosity at saturation (A_{sat}, $cm^3\ g^{-1}$). C: unplanted control, E: Earthworms, L: leek, P: petunia, A: AMF, *df* degrees of freedom. **.** $P< 0.1$, *$P<0.05$, ** $P<0.01$, *** $P<0.001$.

Treatment		K_{str}	ρ(SL)	W_p(SL)	V_p(SL)	A_p(SL)	V_s(SL)	ρAE	W_p(AE)	V_s(AE)	ρ(sat)	A_{sat}
C		0.139 (0.034)	1.289 (0.021)	0.045 (0.008)	0.054 (0.007)	0.008 (0.001)	0.345 (0.009)	1.287 (0.020)	0.060 (0.006)	0.340 (0.009)	1.245 (0.022)	0.196 (0.013)
E		0.090 (0.008)	1.398 (0.039)	0.043 (0.013)	0.053 (0.012)	0.010 (0.002)	0.286 (0.017)	1.396 (0.039)	0.061 (0.011)	0.280 (0.016)	1.346 (0.034)	0.135 (0.020)
L		0.074 (0.011)	1.228 (0.088)	0.039 (0.007)	0.053 (0.004)	0.014 (0.004)	0.393 (0.065)	1.225 (0.087)	0.063 (0.003)	0.385 (0.066)	1.185 (0.084)	0.246 (0.057)
L+E		0.073 (0.015)	1.299 (0.061)	0.044 (0.002)	0.058 (0.002)	0.014 (0.004)	0.337 (0.039)	1.296 (0.061)	0.069 (0.004)	0.329 (0.041)	1.257 (0.063)	0.189 (0.030)
L+M		0.090 (0.035)	1.180 (0.041)	0.046 (0.003)	0.053 (0.003)	0.007 (0.001)	0.419 (0.027)	1.179 (0.041)	0.058 (0.003)	0.415 (0.028)	1.133 (0.049)	0.253 (0.022)
L+M+E		0.089 (0.014)	1.265 (0.046)	0.048 (0.011)	0.061 (0.009)	0.013 (0.003)	0.354 (0.026)	1.262 (0.046)	0.071 (0.008)	0.346 (0.027)	1.214 (0.048)	0.209 (0.027)
P		0.057 (0.012)	1.356 (0.109)	0.036 (0.001)	0.048 (0.003)	0.011 (0.002)	0.321 (0.059)	1.354 (0.108)	0.056 (0.004)	0.315 (0.060)	1.320 (0.104)	0.187 (0.055)
P+E		0.053 (0.018)	1.330 (0.027)	0.037 (0.004)	0.045 (0.005)	0.008 (0.001)	0.330 (0.018)	1.329 (0.026)	0.051 (0.005)	0.325 (0.019)	1.294 (0.028)	0.172 (0.017)
P+M		0.041 (0.006)	1.313 (0.024)	0.038 (0.005)	0.054 (0.006)	0.016 (0.005)	0.331 (0.019)	1.309 (0.023)	0.066 (0.008)	0.321 (0.021)	1.279 (0.025)	0.185 (0.017)
P+M+E		0.073 (0.019)	1.327 (0.043)	0.055 (0.010)	0.064 (0.009)	0.009 (0.001)	0.313 (0.024)	1.326 (0.043)	0.071 (0.009)	0.307 (0.024)	1.283 (0.046)	0.165 (0.020)
ANOVA F-value	*df*											
AMF	1, 20	0.43	1.54	1.55	2.21	0.03	0.68	1.57	2.40	0.64	1.67	0.59
Plant species	2, 20	**6.18 ****	**2.80 .**	0.46	0.40	0.91	**2.69 .**	**2.83 .**	0.42	2.50	**3.18 .**	**3.00 .**
Earthworm	1, 20	0.01	2.01	0.96	1.01	<0.01	2.91	2.04	0.89	2.79	1.70	**3.90 .**
AMF x Plant species	1, 20	0.11	0.05	0.16	1.42	**4.24 .**	0.25	0.04	2.94	0.33	0.07	0.17
AMF x Earthworm	1, 20	2.25	<0.01	0.75	0.91	0.16	0.02	<0.01	0.90	0.02	0.01	0.10
Plant species x Earthworm	2, 20	0.30	0.94	0.21	0.07	**3.65 ***	0.81	0.92	0.44	0.91	0.92	0.39
AMF x Plant species x Earthworm	1, 20	0.48	0.03	0.77	0.30	1.12	0.03	0.03	0.06	0.01	0.02	0.04

is required to better understand these curves, which is not the aim of the present study. We consequently decided to focus our analyses on the specific plasma porosity, water and air content at SL and AE and on the specific structural porosity at SL, AE and saturation (i.e. -10hPa) (see below). As a measure of the hydro-structural stability K_{str} is kept in the analysis.

Shrinkage curves and pore-size distribution

Fig. 5.5 shows the shrinkage curves of the whole dataset. Fig. 5.5a shows that earthworm treatment curves (E) resulted in smaller specific volume than the control (C), meaning a compaction (higher soil density) in the presence of earthworm without plants. Overall, the specific volume of every sample was comprised between 0.7 and 0.9 $cm^3\ g^{-1}$, except for two samples (see Fig. 5.5b and d). In the presence of plants (continuous and dotted black lines on Fig. 5.5b-e), we observed that ShC of similar treatments were not very close to each other, leek samples showing usually a greater variation among a treatment compared with petunia samples. In comparison with petunia samples, the specific volume of leek samples was marginally greater. This was particularly obvious when AMF was added to the treatment (Fig 5.5d). In this case, L+A samples presented larger specific volume than the unplanted control and the P+A samples stood at a similar specific volume than the unplanted control. The result was less obvious when earthworms were added with AMF (Fig. 5.5e).

The respective shrinkage properties derived from the XP model parameters at SL, AE and at saturation (-10 hPa) are given in Table 5.3. A significant effect of plant species was observed on K_{str}. K_{str} decreased in the following order: unplanted > leek > petunia. On the whole, the specific plasma porosity (V_p), the plasma water content (W_p), the specific air content of the plasma (A_p) at SL and AE and the specific structural porosity (V_s) at AE were not affected by the treatments (Table 5.3). However, the bulk density (calculated as the inverse of the specific bulk volume) was marginally affected by plant species at SL, AE and at saturation (-10 hPa). The specific bulk density of leek samples was smaller compared with both Petunia and unplanted samples. Interestingly, the specific air-filled porosity at saturation (A_{sat}) was marginally decreased with earthworms, but marginally greater in the presence of leek (0.22 ± 0.02 $cm^3\ g^{-1}$) compared with petunia (0.18 ± 0.01 $cm^3\ g^{-1}$) and the control (0.17 ± 0.02 $cm^3\ g^{-1}$).

Fig. 5.6 presents the cumulated volume of the pore-size distribution of the different treatments. For a better understanding, we took into account the air-filled porosity at saturation (A_{sat}), corresponding to the coarser (larger than 150 µm) pore volume (Table 5.3). Overall, the pore size radius varied between 2.2 and 125.7 µm. The pore volumes of the E treatment were generally smaller at every pore size than the control (Fig. 5.6a). Moreover, the curves of the unplanted microcosms (grey lines) were rather flat, thus indicating there were only small pore volumes at every pore size. On the contrary, by comparing the different treatments having plants in the microcosms (Fig. 5.6b-e), we observed that leek and petunia curves were more sloppy. This indicates an increased desorbed pore volume at smaller pore radii. In addition, the cumulated pore volumes with leek were usually similar or greater than the control at any pore size, whereas an opposite trend was observed with petunia, at least at higher

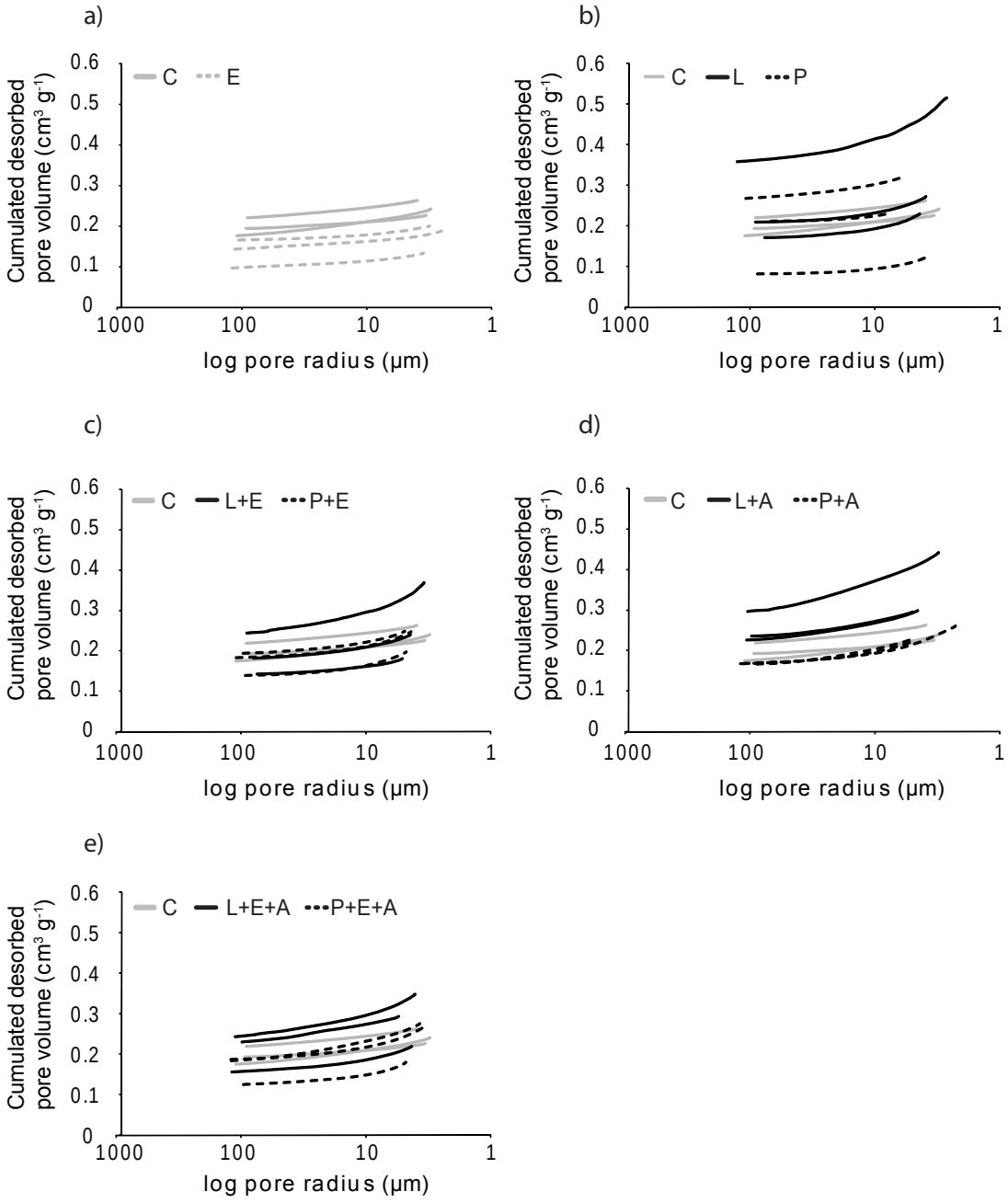

Fig. 5.6 Cumulated desorbed pore volume vs. equivalent pore diameter according to the initial air filled porosity (pore radius > 150 µm). Unplanted treatments with earthworm (E, grey dotted lines) in comparison with the unplanted control (C, grey lines) are represented in (a), the plant treatments: leek (L) and petunia (P) in (b), the plant + earthworms treatments (L+E and P+E) in (c), the plant + AMF (A) treatments (L+A and P+A) in (d) and the plant + earthworms + AMF (L+E+A and P+E+A) in (e). For a better understanding of the figure, leek plant treatments, independently of the presence of earthworms and/or AMF, are represented with black lines and petunia plant treatments with black dotted lines. The control (C) is shown everywhere (b-e) in order to compare the effects of plant species with unplanted microcosms.

pore radii. This is particularly obvious when AMF were present in the microcosms (Fig. 5.6d). As previously described with the ShC (Fig. 5.5), variations among replicates were observed. Moreover, the total cumulated pore volume was significantly different between the plant species treatments ($F_{2,20}$= 3.70, P = 0.04). The total cumulated pore volume of the leek treatment (0.31 ± 0.03 cm^3 g^{-1}) was greater than the petunia (0.23 ± 0.02 cm^3 g^{-1}) and the unplanted control (0.21 ± 0.02 cm^3 g^{-1}). Finally, compared with the air-filled pore volumes, the specific volume occupied by the roots were about 2 or 3 orders of magnitude smaller.

Discussion

Effects of AMF and earthworms on plant growth

Overall, plant growth of leek and petunia was good. Despite a low mycorrhization rate similar for leek and petunia, the presence of AMF enhanced the shoot and root biomass. These results are in accordance with the climate chamber experiment of Milleret et al. (2009) who showed similar results that were explained by an increased P nutrient uptake by the leek roots in the presence of AMF. Despite earthworms are known to beneficially affect plant growth (Scheu 2003), no effect on plant biomass was observed in the present experiment. The results may be explained by the high earthworm mortality that occurred during the experiment. However, contrasting results are found in the literature when considering the effects of earthworms on plant growth, especially in studies where earthworms are mixed with AMF (Eisenhauer et al. 2009; Wurst et al. 2004; Yu et al. 2005). Finally, despite no interactive effects measured between earthworm and AMF, both organisms had opposite effects on plant root architecture. While AMF enhanced the specific root length of plants, earthworms decreased their specific root volume.

Comments on experimental shrinkage curves

To our knowledge, shrinkage results have not been published yet on a silt loam Luvisol. Observations of the whole experimental shrinkage curves showed that three different curve shapes were obtained. Such results are not entirely understood. Although we decided to focus our attention on the SL, AE and saturation part of the curves we found three main reasons that may explain the results. First, the observed curve shapes may be an artefact due to anisotropic volume changes. Boivin (2007) described that crack openings or closings as well as a one dimensional (1-D) vertical collapse may affect the conversion of 1-D height change to volume change (see equation 1). However, first results on this soil confirmed a geometry factor of 1-D measurements close to 3, meaning isotropic shrinkage (Lamy, pers. comm.), and no large cracks have been observed. Second, according to Braudeau et al. (1999; 2004) interpedal swelling may have occurred, as suggested in Fig. 5.4b. However, this phenomenon was mainly observed in ferralitic and Vertisol. Moreover, Braudeau et al. (2004) described a slope of the interpedal phase near 1 and in the part of the curve near saturation when the suction is smaller than -10hPa (Boivin et al. 2006). In our experiment, the slope of this part of the curve was smaller and occurred in a range of potential greater than -10 hPa. The third hypothesis is that contrarily to other soils where shrinkage curve modelling has been successfully applied, the XP model cannot be fitted with unstable soils sensitive

to crusting like the one used in the present experiment.

Effects of plant roots, AMF and earthworms on soil physical properties

Leek and petunia differently affected soil physical properties. The presence of leek in the microcosm marginally increased the specific bulk soil volume (i.e. decreased the bulk soil density) compared with the petunia, despite a great variability among replicates of a single treatment. Additionally, total cumulated desorbed pore volume and the air-filled porosity at saturation (representing the pore radii greater than 150 µm) tended to be greater in the presence of leek compared with petunia or the unplanted microcosms. However, as revealed by Milleret et al. (2009) the effects of roots seem to be negligible as the pore volume generated by both plant species was several orders of magnitude larger than the volume occupied by the roots themselves. Moreover, most part of the root diameters were in the range of pore diameter greater than 150 µm, which corresponds to air-filled porosity at saturation (-10 hPa). According to Milleret et al. (2009), root exudates may have served as carbon source for bacteria that in turn would have enhanced soil aggregation and porosity. The present results therefore also support the idea of a self-organisation of the soil-plant-microbe complex as suggested by Young and Crawford (2004).

In parallel, K_{str} was lower in the presence of plants, suggesting that petunia and leek increased the hydro-structural stability of the soil compared with the control (Schaffer et al. 2008). By measuring the percentage of macroaggregates stability ($WSA_{1\text{-}2\ mm}$) we also found greater soil stability when plant roots were present in the microcosms compared with the unplanted microcosms. These results are in accordance with previous studies, showing a better soil structural stability in the presence of plant roots (Hallett et al. 2009; Milleret et al. 2009). Furthermore, the structural stability of the soil was greater with petunia compared with leek roots. Miller and Jastrow (1990) and Carter et al. (1994) in Six et al. (2004) suggest that root morphology is important and determine the role of root penetration and its influence on soil aggregation. For example, Rillig et al. (2002) demonstrated that $WSA_{1\text{-}2\ mm}$ was different according to plant species from a similar grassland but differing in their functional role (forbs, grasses and legumes). The petunia root network was thinner, more branched and homogeneous than the leek root network (visual observation). This is confirmed with the results of the root biomass and root length of both species. We therefore suppose that leek root architecture (greater mean root diameter, less branched) increased the specific bulk soil volume and porosity, while the petunia root network (greater volume of small root diameter) increased soil stability with a better physical root enmeshment of soil. Furthermore, roots are also known to act as binding agent by releasing organic material within the rhizosphere. They may consequently affect soil stability directly or indirectly (through microbial stimulation) (Six et al. 2004). Such measurements have not been performed in the present experiment. Further analyses of root morphology and root exudates of both species would be useful to better describe such results.

On the whole, AMF induced an increase of the percentage of water-stable macroaggregates ($WSA_{1\text{-}2\ mm}$) as previously described by several authors (Jastrow et al. 1998; Rillig and Mummey 2006; Rillig et al. 2002). Despite the ShC of

the samples containing plant + AMF showed a greater specific soil volume when AMF was grown with leek than with petunia, no significant effect of AMF on the K_{str} and the specific bulk soil density has been measured. This trend was also observed in the pore volume graph (Fig. 5.6). The mycorrhization rate was low in the present experiment. It seems consequently that the presence of plant rather than the presence of AMF have the greatest impact on increasing soil stability, as previously described by Hallett et al. (2009).

At any water content, the presence of earthworm without plants decreased the specific bulk volume of the soil (i.e. increased the soil density). These changes went with a decrease of the pore volume at any pore size. At saturation, the specific bulk soil density was 1.35 g cm^{-3} in the presence of earthworms and corresponds to initial bulk density measured in the field (Chenu, pers. Comm.). The effect of earthworms was however reduced when plants were added in the microcosm. In such cases, plant roots decreased the specific bulk soil density. Again, this is in agreement with the previous result of Milleret et al. (2009) with the same earthworm species but using a soil with a better structure and ten-fold more stable soil.

Conclusions

By using shrinkage analyses combined with biological and chemical measurements, our work highlights the impact of soil organisms on soil physical processes and feedback on plant productivity. This work confirmed the improvement of plant production in the presence of AMF, but no direct effect of earthworms or interactions with AMF were measured. In comparison with a previous experiment (Milleret et al. 2009), and by using a unstable soil with different physic-chemical properties, shrinkage analyses confirmed that i) soil organisms were able to modify soil physical parameters and ii) root architecture was an important factor controlling soil stability. Furthermore, while the compacting effects of the endogeic earthworm species used in the unplanted microcosms and the decompacting and stabilizing effects of plant roots have been shown, the effects measured in the present experiment are less obvious than the results obtained with a more stable soil. Our results, therefore, support the idea that soil type is very important and that the soil response is varying both in function of soil organisms and soil type.

Acknowledgments

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Additional results

To compare the results of this chapter with chapter 2 and 3 (see the general discussion of chapter 7), available P was measured at the end of the experiment. Available P was extracted according to Olsen et al. (1954) as previously described in the Material and Method section of chapter 2 and 3.

Available P in the soil was significantly affected by the presence of AMF ($F_{1,20}$= 21.95, $P < 0.001$) and plant species ($F_{2,20}$= 24.61, $P < 0.001$). As shown in Fig. 5.7, available P concentration in the bulk soil was lower in the presence of AMF (61.49 ± 1.24 µg g^{-1}) than in the absence of AMF (56.21 ± 1.74 µg g^{-1}). Available P concentration was higher in the leek treatment (62.11 ± 0.99 µg g^{-1}) and the control (64.32 ± 1.02 µg g^{-1}) compared with the Petunia treatment (54.18 ± 1.22 µg g^{-1}).

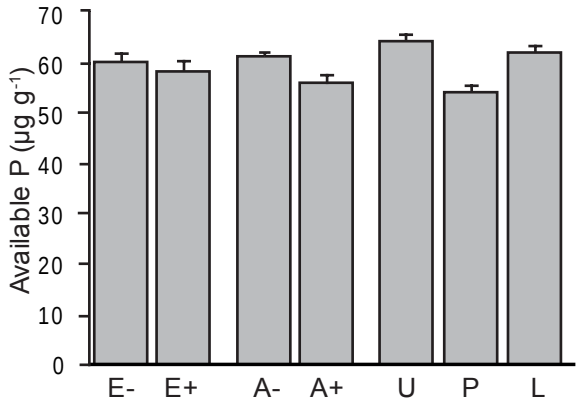
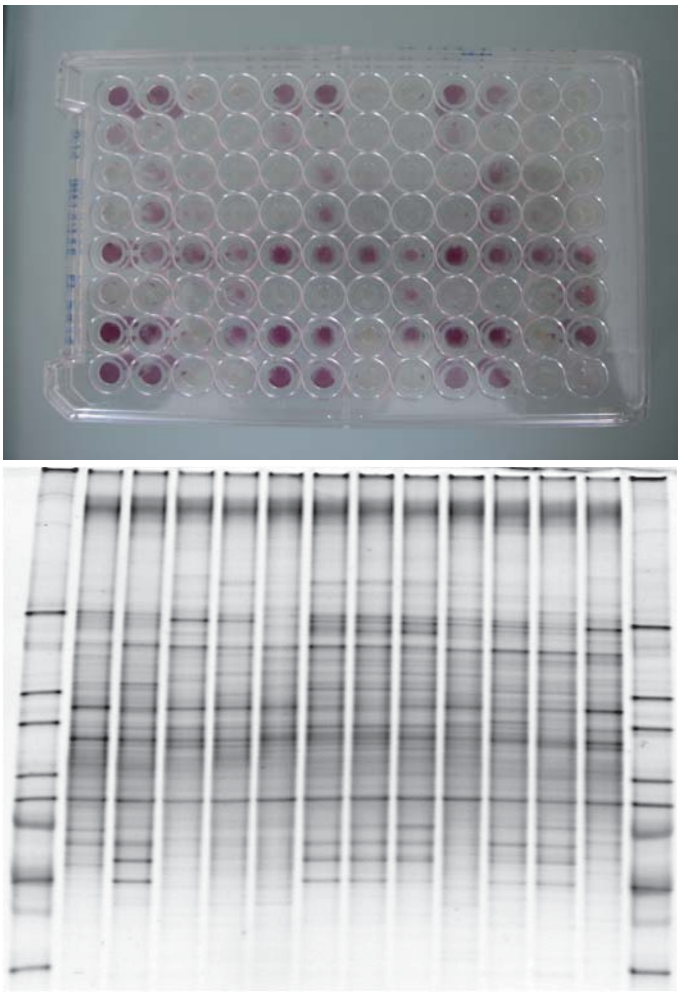


Fig. 5.7 Main effects of the presence of earthworms (E, *Allolobophora chlorotica*), AMF (A, *Glomus intraradices*) and plant species (Unplanted: U, petunia: P and leek:L) on available P (µg g^{-1}). Bar represents mean + SE.



6

The influence of plant roots, earthworms and mycorrhizae on genetic and functional structures of bacterial communities

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Submitted

Abstract

Among the wide range of soil organisms, plants, arbuscular mycorrhizal fungi (AMF), and earthworms are key players in soil processes and as such exert a profound influence on the bacterial communities living in this environment. While their single effects have been widely studied, the combined influence of all three organisms on bacterial communities is still poorly understood. A compartmental experimental design was used, under controlled conditions, in order to investigate the single and combined effects of earthworms (*Allolobophora chlorotica*), AMF (*Glomus intraradices*) and leek roots (*Allium porrum*) on genetic and functional structures of bacterial communities using molecular (16S rDNA-based DGGE) and culture-based (Biolog™ Ecoplate) techniques. These effects were investigated in three different soil fractions termed rhizosphere (fraction of soil influenced by the roots), drilosphere (fraction of soil influenced by earthworms) and the remaining soil (i.e. bulk soil) in a 35-week experiment. When comparing the effects of the different treatments in the bulk soil, the observed variations in the DGGE-based community structures were explained at 10.2% by the presence of roots in the microcosms and at 8.2% by the AMF x leek roots interaction. By using Biolog™ Ecoplate, the bacterial structures were explained at 33.2% by the presence of AMF, followed by the interaction of leek roots and AMF (24.1%) and leek roots (14.9%). Bacterial community structures were not affected by earthworms in the bulk soil. However, when comparing the different soil fractions (drilosphere, rhizosphere and bulk soil), the bacterial communities were different between the drilosphere soil and the two other soil fractions (rhizosphere and bulk soil). In conclusion, AMF, earthworms and roots significantly affected bacterial community structures and the influences of these different soil actors and of their interactions were not limited to their close surrounding soil.

Keywords

DGGE, Biolog™ Ecoplate, drilosphere, rhizosphere, bulk soil, soil interactions

Introduction

Soil bacteria as primary decomposers and important soil enzymes producers, or as plant growth promoters, play an important role on soil fertility and plant health (Tarkka et al. 2008). Regarding the soil functioning, bacteria are included in complex interaction webs (e.g. trophic web) in dynamic equilibrium(s). Sure enough others soil organisms, as plants *via* their root system, arbuscular mycorrhizal fungi (AMF) and earthworms, are also key players in soil processes and interact directly or indirectly with bacteria (Trevors and Van Elsas 1997; Wardle 2002), influencing as such soil bacterial communities.

Regarding the soil trophic food web, soil organisms depend mainly on plants as a primary producer. The soil directly influenced by root activities, the rhizosphere soil, is often considered as a hotspot of microbial interactions as exudates released by plant roots (for details see Jones et al. 2004; Neumann and Römheld 2001) are a main energy source and/or electron donors for microorganisms, and a driving force of their population density and activities. Rhizosphere bacterial communities are therefore often different from those observed in the bulk soil (e.g. Kent and Triplett 2002; Smalla et al. 2001; Soderberg et al. 2002; Weisskopf et al. 2008).

In the same way, it has been shown that, due to their close relationship with plant roots, AMF modify soil bacterial community structures by activating or suppressing some bacterial taxa (Andrade et al. 1997) or activities (Artursson et al. 2005). These effects of AMF may be explained by the presence of fungi themselves, but also by changes in root exudates caused by the symbiosis and the carbon demand of the fungus to the plant (Marschner and Baumann 2003). The external hyphae of AMF extend the influence of fungi on bacterial communities to bulk soil e.g. through their own secretion or the translocation and release of photosynthetically derived carbon compounds (Toljander et al. 2007).

Finally, earthworms, as ecosystem engineers, have a great impact on organic matter decomposition, soil aggregation and, consequently, on soil bacterial communities (Edwards and Bohlen 1996). Earthworms ingest soil particles and organic matter that are modified through the passage in their gut (Brown et al. 2000). The resulting casts or burrows, constituting the drilosphere soil, have been shown to host bacterial communities with a particular structure and composition (Amador and Gorres 2007; Savin et al. 2004). Moreover, earthworms selectively feed on microorganisms, inducing changes in the size or structure of microbial communities (Brown et al. 2004).

The effect of plant roots, AMF or earthworms on bacterial community structures was already studied for each organism separately (Marschner et al. 2004; Scheu et al. 2002; Soderberg et al. 2002) or for the interaction between AMF and plant roots (Marschner and Timonen 2005; Soderberg et al. 2002). However, the combination of the three organisms in a single experiment is still poorly studied. To our knowledge only one paper focused on the effects of the combination of earthworms and AMF on bacteria by using soil microbial biomass (SMB) measurements (Zarea et al. 2009). The aim of our study was consequently to investigate, in a long-term (35 weeks) microcosm experiment, the single or shared effects of plants, AMF and earthworms on bacterial communities. As roots, AMF and earthworms profoundly influence their surrounding soil, bacterial community

structures have been examined in three different soil fractions: rhizosphere, drilosphere and bulk soils. To prevent misunderstandings, the term "hyphosphere" is not employed in the present study, but referred to bulk soil with AMF in comparison with bulk soil without AMF.

Literature reports a high amount of techniques to study soil bacterial communities (Kirk et al. 2004). It has been demonstrated that total community structure with DNA-based community profiles is not sufficient to have a complete understanding of the structure or function of bacterial communities (Jossi et al. 2006; Weisskopf et al. 2008). Most authors suggest combining total community structure analyses with functional analyses (Torsvik and Ovreas 2002). In the present study, two different techniques were used to characterize bacterial community structures. A molecular technique based on DNA fingerprinting called PCR-DGGE (polymerase chain reaction–denaturing gradient gel electrophoresis) was first used in order to have the genetic bacterial structure. This method allowed assessing the shifts among most abundant populations of the global communities (active and non active communities taken together). Second, the functional structure of bacterial communities was estimated by using a common culture-dependent technique based on carbon substrate utilisation profiles (Biolog™ Ecoplate) (Garland 1997). The Biolog™ Ecoplate technique was successfully used in previous studies to determine land use changes (Kohler et al. 2005) or the effect of management practice (Govaerts et al. 2007) on microbial carbon sources utilisation, despite various limitations reviewed in Preston-Mafham et al. (2002).

The two main objectives of this study were to i) explore the individual or combined effects of earthworms (*Allolobophora chlorotica*, Savigny), AMF (*Glomus intraradices*, Schenk & Smith) and leek roots (*Allium porrum*, L.) on the genetic and functional structures of soil bacterial communities and ii) evaluate if different soil fractions, i.e. rhizosphere, drilosphere or bulk soil, from a single experiment harbour similar or specific bacterial communities.

Material and methods

Experimental setup

A compartmental microcosm design was used. It consisted of a PVC tube (35 cm height and 15 cm internal diameter) separated vertically into two equal parts by a nylon mesh (25 µm, ©SEFAR, Switzerland). Each side of the microcosm was filled with six successive 5 cm thick layers of soil remoistened at 22% (w:w) water content. In order to eliminate microorganisms (especially AMF and bacteria), microcosms were first sterilized with γ-irradiation (between 42 kGy and 82 kGy; Studer Hard, Dänikon, Switzerland) and stored at 4°C (McNamara et al. 2003). After soil sterilization, a control microcosm was checked for the presence of cultivable bacteria and fungi. Soil samples were crushed in a mortar with sodium phosphate buffer (0.1 M, pH7) then ten-fold serially diluted and spread in triplicate on Malt Agar and modified Angle media (Angle et al. 1991). No growth was observed after four days of incubation at room temperature. Thereafter, sterilised soil of each microcosm was re-inoculated with 20 ml of a soil suspension containing microorganisms without AMF. This Soil suspension without AMF was obtained by filtering on 11 µm paper (Koide and Li 1989) 100 g of soil dispersed in 1000 ml of autoclaved distilled H_2O. The same soil suspension was used to inoculate all the microcosms.

Eight treatments were defined and represented all possible combinations of the presence/absence of the three following factors: Leek plant (L, *Allium porrum* var. Mercure), AMF (A, *Glomus intraradices*) and earthworm (E, *Allolobophora chlorotica)*. Treatments are abbreviated as follow further in the text ($L^{+/-}A^{+/-}E^{+/-}$), depending of the presence ($^{+}$) or absence ($^{-}$) of each factor. According to the experimental design, the nylon mesh, separating the microcosm into two parts, retained the roots and earthworms but allowed fungal hyphae to pass through. Treatments were attributed as follows to the microcosms. First, one side of each microcosm (allocated at random) received three leek plantlets that were previously sown in sterile conditions. Consequently each microcosm presented two treatments one with and the other without leek plants. Then, the four combinations of the remaining factors, AMF and earthworms, were allocated to the microcosms. In treatments with AMF, inoculums were prepared by mixing 30 g of culture sand substrate with *Glomus intraradices* spores and hyphae, treatments without AMF received 30 g of an inoculum sterilized by autoclaving at 121°C over 1h and gamma-irradiated. The fungi were inoculated (before introducing the leek plantlets) in one side of the microcosm but colonized both sides by passing through the nylon mesh. This compartmental design permitted to separate the individual effect of AMF from the root effect. Finally in treatments with earthworms (E), five earthworms of equal size (total biomass of 1.3 ± 0.1 g) and relieved of their gut contents were added to both sides of the microcosms. This corresponds to a density of 150 g m^{-2}, which is 1.5 times higher than in a maize crop according to Le Bayon and Binet (1999). This endogeic species was selected as such earthworms inhabit the organo-mineral soil horizon, feeding on the soil organic matter closely linked to the mineral matrix and largely burrow within the upper centimetres of the soil (Lee and Foster 1991). As a result, each microcosm received two treatments and each treatment was produced in triplicates, resulting in 12 microcosms (i.e. 24 samples). All microcosms were kept in a climate chamber (Normoflex, KR 11C/200S10, Schaller Uto AG, Bern, Switzerland) under the following conditions: photoperiod 16/8 h (day/night), temperature 18 ± 2 °C and 50% humidity. Microcosms were randomly redisplayed in the climate chamber every week and watered twice a week using a modified Hoagland's nutrient solution without P in order to promote the AMF-plant symbiosis. Every three weeks, each microcosm was adjusted to equal soil water content with deionised water by weighing.

Harvesting

After 35 weeks, three different soil fractions were removed from the microcosms: (1) Rhizosphere Soil (RS), corresponding to the soil still adhering to the roots after gentle shaking, was collected by rubbing roots carefully on a 2 mm mesh sieve; (2) Drilosphere Soil (DS) was obtained by sampling faeces and the few millimetres-thick layer around the earthworm burrow-linings; (3) the Bulk Soil (BS), that consists of the remaining soil fraction not previously sampled as drilosphere or rhizosphere soil, was thoroughly homogenized. All the RS and DS samples as well as a half of each BS samples were immediately frozen at -80 °C for DGGE analyses. The other half part of each BS samples was stored at 4°C and used in the next two days for Biolog™ Ecoplate analyses. RS and DS were retrieved in too low amounts and were consequently not used for Biolog™ analyses.

DGGE fingerprinting

DNA extraction (bead-beating technique using a FP120 FastPrep™ cell disruptor, Savant Instruments), PCR, as well as DGGE (Denaturating Gel Gradient Electrophoresis) were performed for all samples as described by Weisskopf et al. (2005). Briefly from each sample, DNA extraction and purification were performed on about 0.5 g of soil material stored at -80°C (see above). Then a PCR amplification of the 16S rDNA V3 region was performed using, the forward universal primer 338f (5'-ACTCCTACGGGAGGCAGCAG-3'), added with a 40 bp GC clamp on the 5' end (Muyzer et al. 1993), and the reverse universal primer 520r (5'-ATTACCGCGGCTGCTGG-3') (Ovreas et al. 1997). DGGE gels were performed with a 8% (w:v) acryl-bisacrylamide (37.5:1, Qbiogene) gel with 30% to 60% linear urea/formamide (AppliChem, Darmstadt, DE) denaturing gradient (100% denaturant corresponds to 40% formamide with 7 M urea). 800 ng of PCR product obtained from each sample, were loaded on the DGGE gel and submitted to electrophoresis at 60 °C with a constant voltage of 150 V during five hours. The gels were stained in the dark during 20 min in 0.01% Sybr Green I (Molecular Probes, Leiden, NL) in TAE buffer 1X (AppliChem), and photographed with the system Geldoc (Fisher Bioblock Scientific, Illkirch, FR). Gel images were normalized according to the reference patterns and the profiles were compared using the GelCompar software (Applied Maths, Austin, USA).

For statistical analyses, DGGE profiles were then converted into two numerical matrices giving for each band in a sample profile a distance of migration and a density in pixels. The first DGGE matrix (DGGE BS) grouped results from the bulk soil samples taken from both sides of the 12 microcosms (24 samples). This matrix was employed in order to test for the effect of the three factors (i.e. presence/absence of leek plants, AMF and earthworms) on the bulk soil bacterial communities. The second matrix (called DGGE SF) grouped results from the different soil fractions (drilosphere, rhizosphere and bulk soil). To allow direct comparison of bacterial community structures between the soil fractions, each side of microcosms had to contain the three samples, i.e. side of microcosm with plants and earthworms had to be used. As a result, the DGGE SF matrix was built by using the three soil fractions sampled in the three replicates of the two treatments $L^+E^+A^+$ and $L^+E^+A^-$, giving a total of 18 samples (2 treatments x 3 soil fractions x 3 replicates). The richness (number of bands), the Shannon diversity and the evenness indexes were calculated from the previously described matrices.

Carbon source utilization profiles

Carbon (C) source utilization profiles were determined with the Biolog™ Ecoplate system (Biolog, Inc., Hayward,m CA, USA) with a procedure adapted from Garland and Mills (1991). First, 1 g of fresh soil was crushed in a mortar and diluted in a sterile sodium phosphate buffer 10 mM pH7 (Na_2HPO_4/NaH_2PO_4) to obtain a final concentration of 10^{-3} colony forming unit per ml (evaluated on modified Angle medium). Then, a 150 µl aliquot was inoculated in each well of Biolog™ Ecoplate. One plate contained 31 different C sources, each present in triplicates. It also included a negative control corresponding to a well without carbon source. Biolog™ Ecoplates were incubated 72 hours in the dark at 15 °C. The carbon sources utilisation is indicated by the reduction of tetrazolium (colourless) to

formazan (purple) during cell respiration. The level of respiratory activity for each well was determined by measuring the optical densities at 630 nm using an automatic microplate reader (Jupiter, UVM 340, ASYS/Hitech, Austria).

To prepare data for statistical analyses, the absorbance value of the control well of each plate was subtracted from absorbance values of the C substrate wells. The average well colour development (AWCD) was calculated among the three replicates by dividing the sum of the absorbance by 31 (number of substrates). The value of each individual well was then divided by the AWCD to compensate for variation in well colour development caused by different cell densities (Garland and Mills 1991). For each substrate, the average of the absorbance values corrected by AWCD was calculated from the three replicates. This calculated variable is referred to as "corrected Abs." in the following text and was used to build the Biolog™ Ecoplate data matrix for multivariate analyses (see below). According to Insam (1997), the 31 C substrates were grouped into five biochemical categories (polymers, carbohydrates, carboxylic acids, amino acids and amines/amides) by summing the "corrected Abs." belonging to a biochemical category.

Statistical analysis

Univariate analyses using ANOVAs were performed for the DGGE profiles on the richness (number of bands), the Shannon diversity index (calculated as $-\sum p_i \log_2(p_i)$ where p_i represents the relative abundance of one given population in the profile) and the Evenness (calculated by dividing the Shannon index by the log of the richness), and for the Biolog™ Ecoplate on the AWCD and the sum of the "corrected Abs." of the five biochemical substrate categories. Partly nested ANOVAs were used in order to take into account that many samples were in the same microcosm. In this case, leek roots were considered to be nested within the microcosm. Consequently, the ANOVA model contained earthworms and AMF as between factors and leek roots or soil fractions as within factors.

Redundancy analyses (RDA) were performed on DGGE and Biolog™ Ecoplate matrices in order to measure the influence of the treatments on bacterial community profiles. First, the matrices performed by using the bulk soil samples (DGGE BS and the Biolog™ Ecoplate matrices) were constrained by seven explanatory variables, i.e. the presence/absence of the three main factors (Leek roots, AMF and Earthworms) and their interactions (LxA, LxE, AxE, LxAxE). Second, the DGGE SF matrix was constrained by the following explanatory variables, i.e. the presence/absence of the AMF factor and the soil fractions (qualitative variables: rhizosphere, drilosphere or bulk soil). Moreover, to assess the importance of each explanatory variable, separated RDAs were calculated for each one. Before the analyses, matrices were first arcsinus transformed and then Hellinger transformed (Legendre and Gallagher 2001) since RDA is not appropriate for the analysis of matrices with a high number of zeros. All analyses were performed using the statistical software package R 2.6.0 (R Development Core Team 2007).

Results

Comparison of the treatments in the Bulk Soil

Genetic structure of bacterial communities

The genetic bacterial community structure was analyzed using 16S rDNA DGGE profiles. The richness of the most abundant bacterial phylotypes in the community (Fromin et al. 2002), given by the number of bands in each DGGE BS profiles, ranged from 16 to 24 per sample and was not significantly affected by the presence/absence of AMF, leek and earthworms. However, diversity (*H'* Shannon diversity index) was significantly greater ($F_{1,17}$=7.30, P=0.02) in BS samples from microcosms with leek (*H'*= 2.89 ± 0.03) than without leek (*H'*= 2.78 ± 0.03) . The evenness was close to 1 and varied between 0.93 and 0.97; this value was also significantly ($F_{1,17}$=33.11, P<0.001) greater in the presence than in the absence of leek.

The RDA ordination plot of the DGGE BS matrix is presented in Fig. 6.1a. The first two axes explained respectively 11.0% and 10.2% of the variation. The first axis clearly separated BS samples in treatments containing leek roots ($L^+A^-E^-$, $L^+A^+E^-$, $L^+A^-E^+$, $L^+A^+E^+$) (in black) from treatments without leek roots (in white). BS samples were not clearly separated by the presence of earthworms and AMF. The percentage and significance of explained variation between BS samples, due to the presence of leek, AMF, earthworms and their interactions, are shown in Table 6.1. The presence of leek and the interaction between leek and AMF (AxL) significantly explained together 19% of the variation among BS DGGE profiles. The presence of earthworms, AMF or other interactions between factors did not explained significant variation among these profiles.

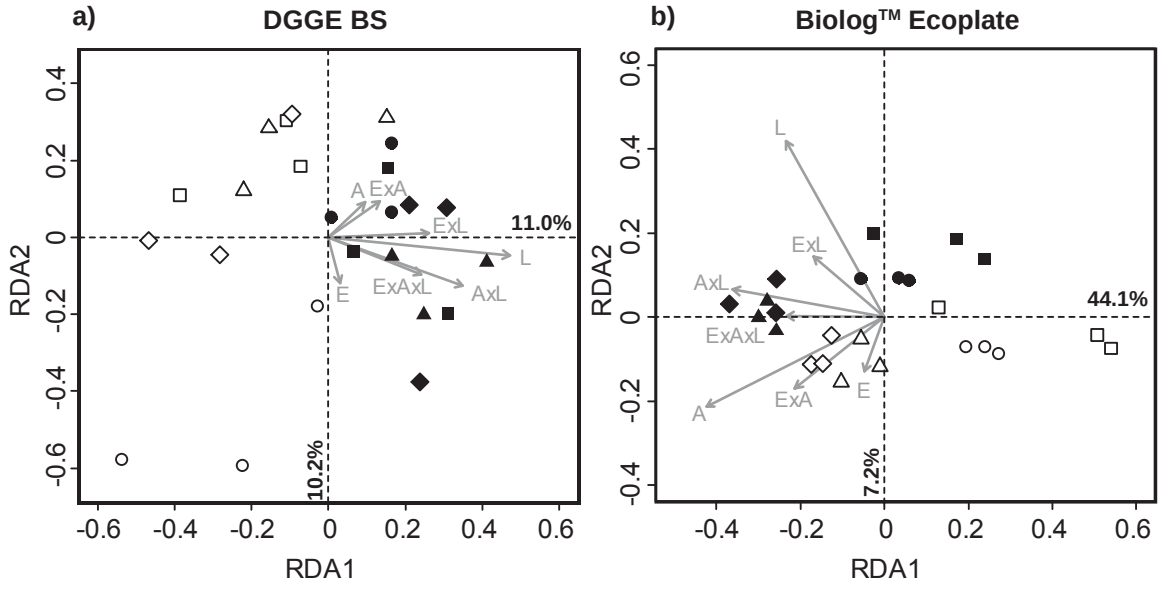

Fig. 6.1 Redundancy analysis plot of the bulk soil matrices based on a) DGGE profiles (DGGE BS) and b) Biolog™ Ecoplates. Open square: $L^-A^-E^-$, black square: $L^+A^-E^-$, open triangle: $L^-A^+E^+$, black triangle: $L^+A^+E^+$, open diamond: $L^-A^+E^-$, black diamond: $L^+A^+E^-$, open circle: $L^-A^-E^+$, black circle: $L^+A^-E^+$. L: Leek, A: AMF, E: Earthworm.

Table 6.1 Redundancy analysis values of DGGE and Biolog™ Ecoplate results from Bulk Soil (BS): Percentage of explained variation using RDAs, P-value and rank of the explanatory variables. E: Earthworm, A: AMF, L: Leek, **P*<0.05, ** *P* <0.01, *** *P* < 0.001.

	DGGE BS			Biolog™ Ecoplate BS		
Variable	explained variation	*P-value*	Rank	explained variation	*P-value*	Rank
E	3.72		7	3.97		7
A	5.58		3	33.21	***	1
L	10.23	***	1	14.92	**	3
ExA	4.12		6	9.99	*	5
ExL	4.51		5	7.48		6
AxL	8.22	*	2	24.12	***	2
ExAxL	5.13		4	10.51	*	4

Table 6.2 Results from partly nested ANOVA testing the effect of earthworms, AMF and leek roots on the average well colour development (AWCD) and the sum of the "corrected Abs." obtained from substrates consumption values of the different biochemical categories in the bulk soil (polymer, carboxylic acid, carbohydrates, amino acid and amine/amid). E: Earthworm, A: AMF, L: Leek roots, df degree of freedom, MS mean square. * *P*<0.05, ** *P*<0.01, *** *P*<0.001.

		Biolog™ Ecoplate											
	df	AWCD		polymer		carboxylic acid		carbo-hydrate		amino acid		amine/amid	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Between microcosms													
E	1	2.93		4.63		1.37		0.04		0.49		0.03	
A	1	49.26	***	0.88		8.34	*	27.44	***	38.04	***	1.05	
E x A	1	5.22		0.82		0.01		0.99		3.78		0.73	
Residuals (MS)	8	$3.50\ 10^{-3}$		0.09		0.42		0.49		0.07		0.22	
Within microcosms													
L	1	3.26		1.49		14.26	**	46.83	***	0.49		10.28	*
E x L	1	0.23		0.09		0.22		1.89		1.08		0.67	
A x L	1	0.28		0.80		1.15		0.08		2.04		5.95	*
Residuals (MS)	9	$5.13\ 10^{-3}$		0.07		0.24		0.12		$8.52\ 10^{-2}$		$3.45\ 10^{-2}$	

Functional structure of bacterial communities

The effect of the three factors (AMF, earthworms, leek roots) and their interactions on functional bacterial structures was examined using Biolog™ Ecoplate substrates consumption analysis. The RDA ordination plot of the Biolog™ Ecoplates matrix, performed with results from BS samples, is represented in Fig. 6.1b. The first two axes explained respectively 44.1% and 7.2% of the variation. The first axis clearly separated the BS samples in treatments with AMF ($L^-A^+E^-$, $L^-A^+E^+$, $L^+A^+E^-$, $L^+A^+E^+$) (diamond and triangles) from those devoid of AM fungi, while the second axis separated treatments with leek roots (in black) from the treatments where roots were absent (in white). Table 6.1 shows that the presence of AMF and the interaction between AMF and Leek (AxL) significantly explained more

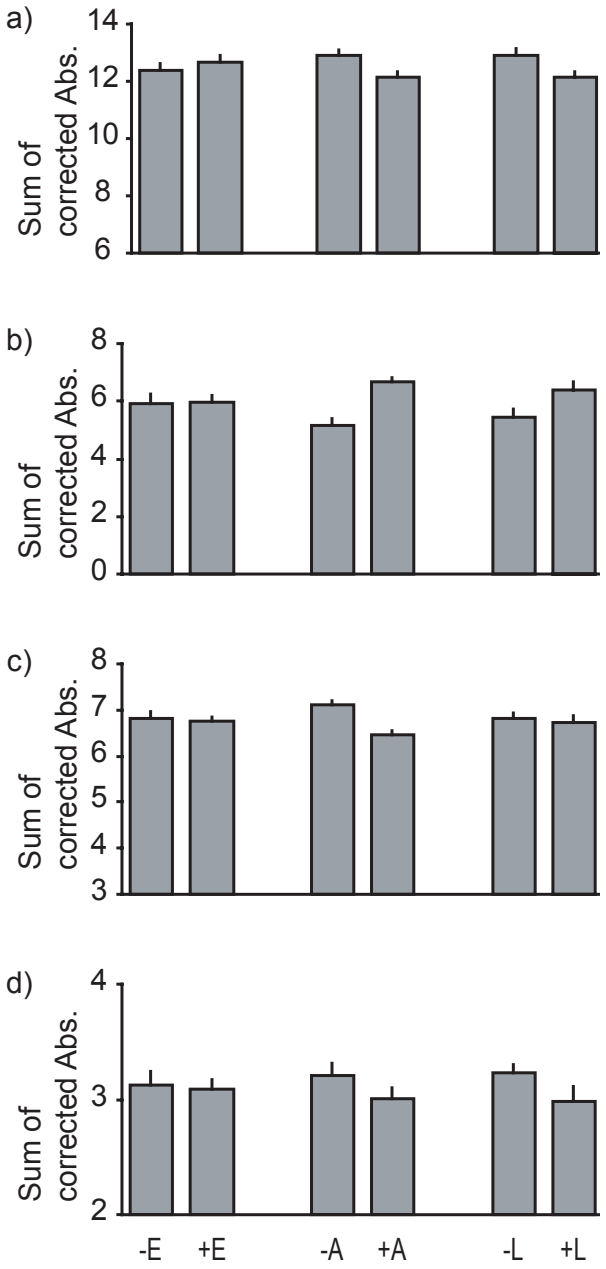

Fig. 6.2 Main effects of the presence of Earthworms (E), AMF (A) and Leek roots (L) on the sum of the "corrected Abs." of the biochemical substrate categories: a) carboxylic acids, b) carbohydrates, c) amino acids and d) amine/amid. Bar represents mean + SE.

than 57% of the variation among Biolog™ Ecoplates profiles. They are followed by the presence of leek (15%), the interaction between earthworms, AMF and leek (ExAxL, 10.5%) and the interaction between earthworms and AMF (ExA, 10%). The average well colour development values (AWCD) ranged from 0.94 to 1.34. AWCD obtained from AMF treated soil samples (Table 6.2) were significantly greater than those without AM fungi, 1.24 ± 0.02 *vs.* 1.07 ± 0.03 respectively. The average microbial consumption of the different categories of biochemical compounds was significantly different for soil samples from AMF treatments (Table 6.2), e.g. carboxylic acid and amino acids were less consumed by AMF treatment samples whereas carbohydrates were it more (Fig. 6.2a, b, c). The carboxylic acid, carbohydrate and amine/amid substrates were used differently by BS microbial communities from leek treatments. Carboxylic acid (as with AMF) and amine/amide were less used, and carbohydrates (also as with AMF) were more consumed (Fig. 6.2a, b, d). Moreover the decreased consumption of amine/amid was accentuated when both leek and AMF were present in the treatment (Table 6.2, AxL, $P < 0.05$). Finally, the presence of earthworms did not significantly affect the microbial consumption of any biochemical substrate categories; and the polymer one was always similarly consumed whatever the treatment (Table 6.2).

Comparison of drilosphere, rhizosphere and bulk soil with or without AMF

Genetic structure of bacterial communities

The number of bands (richness) present in the DGGE SF profiles ranged between 17 and 23 per sample. The Shannon diversity index and the evenness index were not significantly different between the three soil fractions and averaged at 3.17 ± 0.02 for H' and at 0.92 ± 0.01 for E (data not shown). The RDA ordination plot of the DGGE SF profiles constrained by AMF and soil fractions is represented in Fig. 6.3. The

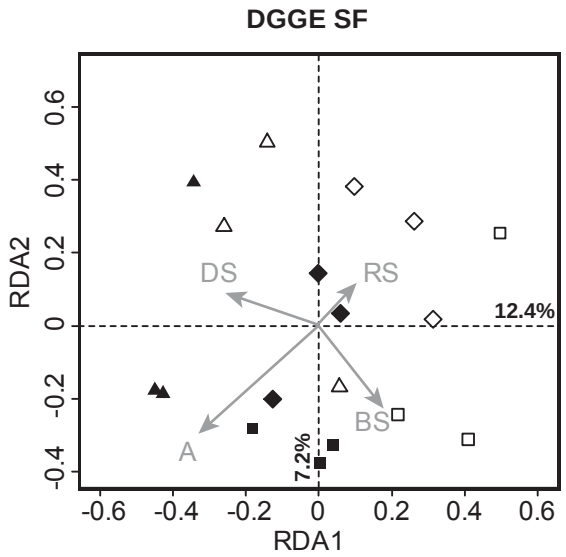

Fig. 6.3 Redundancy analysis plot of the DGGE profiles based on the soil fraction DGGE matrix (DGGE SF). Square: bulk soil (BS) sampled in the two treatments $L^+A^+E^+$ and $L^+A^-E^+$, triangle: drilosphere soil (DS), diamond: rhizosphere soil (RS). Open; without AMF, black: with AMF. L: Leek, A: AMF, E: Earthworm.

first two axes explained respectively 12.4% and 7.2% of total variation. The first axis opposed DS samples on the left and the two other RS and BS fractions on the right. Bacterial community structures were significantly different in DS, RS and BS fractions (explained variation = 16.3%, $P = 0.05$) and were only marginally modified by the presence of AMF in the treatments (explained variation = 9.1%, $P = 0.06$).

Discussion

Effect of plant roots, AMF and earthworms on bacterial communities in the bulk soil

In the bulk soil, the presence of leek plants exerted an effect both on genetic and functional structures of bacterial communities at a distance from the rhizosphere, since significant differences were found comparing soil community profiles derived from treatments with and without plants. Moreover, the populations' diversity and evenness of the bulk soil communities were higher in microcosms with *Allium porrum*. Such results have been also observed in a 168-day microcosm experiment planted with Salix *sp*. by de Cárcer et al. (2007). In this paper, the authors suggest that bacterial communities in a bulk soil influenced by a rhizosphere soil also benefit, even if indirectly, from the plants activities. Theoretically, bulk soil is defined as the soil fraction not penetrated/influenced by roots. In practice, we paid particular attention in separating the rhizosphere soil from the bulk soil at the time of harvest. Such collected bulk soil, as consequence of the long time of the experiment (245-days) and the limited volume of the microcosms (around 6 L), has unavoidably been influenced by roots e.g. by earlier root passage, secretion, decay or grazed by earthworms. Field or pot experiment comparing rhizosphere and bulk soil without unplanted control should therefore be interpreted cautiously, bearing in mind a potential influence of earlier roots in the soil later to be sampled as bulk soil.

Considering the AMF influences on bacterial communities in bulk soil, our results differed according to the method chosen to monitor them: not significant using 16s rDNA - DGGE profiles, and significant using carbon source utilization profiles. This was not really surprising, and it is well accepted today that molecular and cultural approaches give complementary informations on a studied object as seen from different points of view: DGGE pointing the abundant and present populations whereas Biolog™ Ecoplate their putative activities (in our case related to C substrate consumption). Biolog™ ecoplates do not evidence actual microbial activities in soil, but this method highlights their reactivity according to the propose substrates, e.g. a compound should quickly be consumed as

some similar ones with the bacterial activities adapted for its consumption were already present in the studied samples, with no need for a long activation time. Nevertheless, both approaches provided results in contradiction with previous studies using either DNA-based DGGE profiles or Biolog™ techniques. First, Marschner and Baumann (2003), using 16s rDNA-based DGGE profiles, found that mycorrhizal colonization induced changes in the bacterial community structure in the bulk soil. By comparing DNA- or RNA-based DGGE profiles in the rhizosphere and the bulk soil, Vestargard et al. (2008) and Marschner et al. (2001) also found significant effect of the presence of AMF on bacterial community structures. Second, Soderberg et al. (2004) showed, by using Biolog GN techniques, that AMF had no effect on bacterial communities in soil planted with leek and concluded about the weaker resolution power of the Biolog technique to differentiate treatments compared to the PLFA one. Our results do not support such technique evaluation, however, in Biolog GN some C sources (D-cellobiose, D-xylose, D-malic acid, L-arginine, 2-hydroxybenzoic acid and 4-hydroxybenzoic acid) were absent compared to Ecoplate (Kirk et al. 2004). The presence of these substrates in Biolog™ Ecoplate may explain difference between both studies. In addition, grouping the 31 C substrates highlighted which biochemical substrate was involved in differentiating the effect of AMF on the functional structure of bacterial communities. Overall, plant and AMF similarly affected bacterial utilization of these categories, except for amino acid consumption. Amino acids, i.e. L-arginine, L-asparagine, L-phenylalanine, L-serine, L-threonine and glycyl-L-glutamic, were less consumed by microorganisms in the presence of AMF whereas no significant effect was observed for the plant treatments. This is in contradiction with Secilia and Bagyaraj (1987) who showed, with a dilution plate method, that bacteria requiring amino acid were stimulated under P-deficient conditions in the mycorrhizal root zone, i.e. in the rhizosphere. However, in our study, amino acid consumption was measured in the bulk soil. We therefore suppose that the impact of AMF on bacterial communities can be related to amino acid consumption that can in turn be different according to the soil fractions. Finally, the obtained results may be explained by external hyphae exudation, as already proposed by Toljander et al. (2007) with an *in vitro* experiment. The presence of AMF and their associated exudates may have modified bacterial community structures or potential activities.

When analysing bacterial communities in a higher level of interactions, both genetic and functional structures of bulk soil bacterial communities were significantly affected by the interaction between AMF and Leek roots. In general, it is assumed that, in the rhizosphere, bacterial response varies among plant species (Marschner et al. 2004), AMF species (Andrade et al. 1997; Marschner et al. 2001), and that interactions between plant species and AMF occur (Marschner and Timonen 2005). AMF colonization modify both qualitatively and quantitatively the release of root exudates (Graham et al. 1981). AMF can therefore affect indirectly the bacterial community structures. In our experiment, the percentage of root colonisation by AMF was high and no AMF was observed in treatment without AMF (Milleret et al. 2009). Root exudates could consequently have been modified. Further analysis on leek root exudates grown with and without AMF would be useful for a better understanding of these results.

Finally, earthworms are often considered as driving factor of the soil microbial community (Brown et al. 2000). However, in this experiment, no effect on bulk soil bacterial genetic and functional structure was evidenced by their presence in a treatment

Effect of soil fractions on bacterial communities

As expected, bacterial communities were differing according to the soil fraction they lived in. As shown on the ordination plot, the community structure of bacteria living in the drilosphere soil was clearly different from the structure of communities living in the rhizosphere and the bulk soil; the latter being separated less clearly (second axis of the RDA). The presence of AMF marginally affected bacterial community composition in all these fractions. In the same experiment (Milleret et al. 2009), nutrient content have been shown to be different in the three soil fractions. Drilosphere soil contained more available P and total N but less total C that the bulk soil or the rhizosphere soil. These results may therefore be explained by the ability of plant roots, AMF and earthworms to modify bacterial structures and activities in the different soil fractions.

Over the past, most studies focused on the comparison between the rhizosphere and the bulk soil. Our results confirmed previous studies, based either on culture dependent or independent techniques, showing that bacterial communities differed between these two soil fractions either in field condition or in pot experiment (Jossi et al. 2006; Kandeler et al. 2002; Smalla et al. 2001; Soderberg et al. 2002).

Moreover, as previously described by Edwards and Bohlen (1996), the particular bacterial community structure in the drilosphere soil can be due to the passage of the soil through the digestive system of the earthworm. Scheu et al. (2002) have also shown, by using a microcosm experiment, that bacterial communities are differently modified by earthworm ecological categories (endogeic *vs* epigeic). Burrowing and casting activities are known to affect soil porosity and soil aeration (Edwards 2004). Drilosphere soil could subsequently have enhanced aerobic communities as suggested by Devliegher and Verstraete (1995) and consequently modified the microbial community structure. These differences between casts and the bulk soil were also observed by Amador and Gorres (2007) for anecic casts after a rain event.

Conclusion

In the present experiment, soil organisms as AMF, earthworms and plant significantly affected bacterial community structures. Overall genetic and functional structures of bacterial communities were mainly affected by individual and interactive effects of plants and AMF in the soil. Earthworms had no direct impact on bacterial communities in the soil, but the casts and burrows they produced (i.e. drilosphere soil) harboured specific communities. More investigations are therefore required to better understand interactions between plants, AMF, earthworms and soil bacteria. In particular a better characterisation of particular bacterial guilds or functional populations that were enhanced under the influence of the three soil organisms and in the different soil fractions would be interesting in order to better understand their effect on soil fertility, plant growth or nutrient uptake.

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7

General discussion

Roxane Milleret

The aim of the general discussion is to give an overview of the research conducted through the three experiments in a synthesized form, but not to repeat the results and the discussions of each previous chapter separately. This general discussion is separated into three parts. The first part describes and discusses the general impacts of roots, AMF and earthworms on the soil properties. The second part mainly focuses on biological interactions between earthworms, mycorrhiza and plants, with a particular attention to individual and/or interactive effect-s of earthworms and AMF on plant production. Finally, the main perspectives of the thesis are discussed.

Effects of earthworms, mycorrhizae and roots on the soil properties

The effects of earthworms, AMF and roots have been measured on different soil properties during this thesis. Table 7.1 highlights the main results from each of the three experiments. This section will first discuss the impacts of AMF, roots and earthworms on soil chemical properties, then on soil physical properties and finally on the communities of soil microorganisms. The last section gives an overview of the main influences of soil organisms on soil properties.

Effects on soil chemical properties

During this work, soil chemical analyses were mainly focused on phosphorus (P) because P is often a limiting nutrient for plant growth. Nitrogen and carbon have also been measured but only for the first experiment. The effects of AMF, earthworms and roots on soil chemical properties are summarized in Table 7.1.

The presence of leek roots in the microcosms significantly decreased available P in the bulk soil in Exp 1 but in Exp 2 and 3, available P in the soil was not different between unplanted and leek-planted microcosms (Table 7.1). Plant growth was limited by a mite infection during Exp 2, which may explain why the roots were less efficient to uptake P from the bulk soil. In the third experiment, the soil used was a silt loam Luvisol. This soil is acknowledged to be unstable and sensitive to crusting and erosion, and therefore less favourable to good biological activities compared to other soils. Results of the third experiment also showed that the effect of plant roots on available P concentration in the

Table 7.1 Effects of roots, AMF, earthworms, their interactions in the bulk soil and soil fractions (drilosphere, rhizosphere and bulk soil) on the chemical, physical and biological properties studied in the three experiments of the thesis. ↑ or ↓: the response variable is significantly increased or decreased ($P < 0.05$) in the presence of the main factors (plant roots, AMF and earthworms). Yes: the response variable is significantly affected ($P < 0.05$) by the explanatory variables, but positive or negative effects cannot be described. Marginal effects ($P < 0.1$) are put into brackets; ns, not significant; -, not available; DS, Drilosphere Soil; RS, Rhizosphere Soil; BS, Bulk Soil; L, Leek plant; P, Petunia plant; U, Unplanted.

Soil properties	Experiment	Chapter	Response variable	Bulk Soil						Soil fractions
				Roots	AMF	Earth-worms	Root x AMF	Root x Worm	AMF x Worm	
Chemical	1	2	Available P	↓	↓	ns	yes	ns	ns	DS>BS=RS
			Total N	ns	ns	ns	ns	ns	yes	DS>BS>RS
			Total C	ns	ns	ns	ns	ns	yes	BS=RS>DS
	2	3	Total P	(↓)	(↓)	ns	ns	ns	(yes)	DS=RS>BS
			Organic P	↓	ns	ns	ns	(yes)	ns	ns
			Inorganic P	ns	ns	ns	ns	ns	ns	DS=RS>BS
			Available P	ns	ns	ns	ns	ns	(yes)	DS=RS>BS
			Acid phosphatase	↑	ns	ns	(yes)	ns	ns	RS>DS>BS
			Alkaline phosphatase	↑	(↑)	ns	ns	ns	ns	DS=RS>BS
	3	5	Available P	L=U>P	↓	ns	ns	ns	ns	-
Physical	1	2 and 4	$WSA_{(1\text{-}2\ mm)}$	↑	ns	↓	yes	ns	ns	-
			Bulk soil density	↓	ns	↑	yes	-	-	-
			hydro-structural stability	↑	ns	↓	-	-	-	-
			structural pore size volume	↑	ns	↓	yes	-	-	-
	3	5	$WSA_{(1\text{-}2\ mm)}$	P>L>U	↑	ns	ns	ns	ns	-
			Bulk soil density	L<P=U	ns	(↑)	ns	ns	ns	-
			hydro-structural stability	P>L>U	ns	ns	ns	ns	ns	-
			structural pore size volume	L>P=U	ns	↓	-	-	-	-
Biological	1	6	Bacterial community structure (DGGE)	yes	ns	ns	yes	ns	ns	yes
			CLPPs (Biolog)	yes	yes	ns	yes	ns	yes	-

bulk soil was different depending on the plant species. Contrarily to leek roots -that showed no effect- less available P was found in the bulk soil in the presence of petunia. This result may be explained by differences between the root system of the two plant species: the petunia root system is indeed thinner and highly branched in comparison with the leek root network (see the results of chapter 5). This was certainly an advantage for petunia plants that were hence able to acquire nutrient in smaller soil pore diameters.

Looking at AMF, they did not significantly affect P availability in the bulk soil of the second experiment but the available P concentration was significantly lower in their presence in the first and the third experiment (Table 7.1). As a general rule, plant roots have been shown to develop many strategies to acquire soil nutrients like the extension of the root network, the secretion of organic acids, the secretion of phosphatase enzymes, the emission of protons, etc. Among them, the mycorrhization infection has been widely developed by terrestrial plants. Our results are in agreement with the general point of view that external hyphae are an extension of the root network that enhances root nutrient uptake in soils, especially in P-limited conditions like in our experiments (Jansa et al. 2005; Marschner and Dell 1994; Smith and Read 1997).

Focusing on earthworms, no significant effect on chemical soil properties was observed in the bulk soil. Earthworms mainly affected these soil properties via the formation of the drilosphere soil through casting and burial activities (Table 7.1). In the two first experiments, P and total N content were enhanced in the drilosphere soil, thus confirming previous results of several studies (Edwards and Bohlen 1996; Le Bayon and Binet 2006; Lee 1985; Sharpley and Syers 1976). Results of the first experiment also highlighted some temporal variations: the nutrient availability in the drilosphere but also in the rhizosphere and in the bulk soil differed after 5, 15 or 35 weeks.

Effects on soil physical properties

Studying the soil structure, two different techniques, one destructive (macroaggregate water stability) and the other non destructive (shrinkage analysis) have been successfully used during the thesis. Similar results have been obtained from both techniques. The effects of earthworms, AMF and roots on soil physical properties are presented in Table 7.1. Roots affected the soil structure by decreasing the soil density and increasing the soil stability. As suggested in chapter 5, the soil structure is also strongly influenced by the root architecture. In the present thesis, AMF had no significant effect on the soil structure, but they significantly and positively interacted with plant roots. Plants had therefore the greatest positive impact on the soil structure, but AMF had rather a synergistic effect by accentuating the effect of plant roots.

It is generally acknowledged that earthworms have a beneficial role on soil structure (Edwards and Bohlen 1996). Most studies compare the effect of casts or burrows with the bulk soil and demonstrate that the soil stability is enhanced in cast, especially after aging and drying (Shipitalo and Protz 1989). In our results by contrast, a de-structuring effect of earthworms was highlighted in experiments 1 and 3 and with two different types of soil. The endogeic earthworms we studied had a negative impact on the soil structure, mainly by decreasing the soil stability and increasing the bulk soil density (i.e. through compaction). As described by Blanchart et al. (1997) for tropical

earthworms, *Allolobophora chlorotica* may behaviour as an endogeic compacting species (see the discussion of chapter 4). Moreover, the soil compaction we observed may be not only due to the species of earthworms, but also to the type of soil material we analysed. Actually, in our experiments, the soil analyses were performed on bulk soil samples for studying the macroaggregate stability or on the entire soil cores for shrinkage analysis; in the literature, such measurements are usually performed on surface casts samples and compared with the non-ingested soils (Brossard et al. 1996; Shipitalo and Protz 1989).

Effects on soil biological properties

During this work, the effect of earthworms, AMF and roots on biological soil properties has been measured by analysing their influence on the structure of the bacterial communities using two techniques: DGGE and community-level physiological profiles (CLPP). Soil bacteria are very important component of the belowground system. They are major factors of the decomposition of dead organic matter, and are a source of food for organisms of higher trophic levels. Studies performed on soil bacterial communities generally compare rhizosphere or drilosphere soil with the bulk soil. To our knowledge, the effects of roots, earthworms or AMF in the bulk soil or the comparison of the three soil fractions in a single experiment has never been investigated yet. Our results show that AMF and roots significantly affected bacterial community in the bulk soil (Table 7.1). This is a very interesting result as it suggests that bacterial communities of the bulk soil are influenced by the proximity of roots or more precisely by the rhizosphere. As suggested in chapter 6, the bulk soil was certainly influenced by roots e.g. by earlier root passage, secretion, decay or grazed by earthworms, especially after 35-week experiment and in a limited soil volume. By contrast, such an effect was not observed for the presence of earthworms in the bulk soil, but distinct bacterial communities were recovered in the three soil fractions. Bacterial community structures inhabiting the drilosphere soil were different from rhizosphere or bulk soil fractions. It is generally agreed that microbial activity (especially the mineralization rate of nitrogen by bacteria) is stimulated in casts and burrows compared with the surrounding soil (Edwards and Bohlen 1996; Lee 1985). Although bacterial populations in our experiment have not been sequenced and the species present are unknown, it is highly possible that bacterial populations implicated in geochemical cycles (e.g. the N cycle) were enhanced. If so, bacterial community structure in the drilosphere could help explaining the increased amount of total N in the drilosphere soil in comparison with other soil fractions (see above the effect of earthworms on chemical soil properties).

Overview of the effects of soil organisms on soil properties

Overall, results confirmed our two first general hypotheses. Earthworms, mycorrhizae and plant roots have various individual and interactive effects on soil properties and these properties were also different in the three fractions of soil.

The general effects of soil organisms on soil properties may be summarized as follow:

i. *Glomus intraradices* principally affects soil **chemical** properties by modifying the soil nutrient content, mainly decreasing the amount of available P in the surrounding soil

ii. *Allolobophora chlorotica* greatly influences soil **physical** properties by destabilizing the soil and increasing the bulk soil density

iii. The roots of *Allium porrum* and *Petunia hybrida* affect both **chemical and physical** soil properties by reducing the P availability in the bulk soil and by improving the soil structure

iv. The structure of the bacterial communities is affected by the three soil organisms.

Biological interactions between the three soil organisms

Biological interactions between earthworms, AMF and plant were measured throughout the three experiments (see details in chapters 2, 3 and 5). Results are summarised in Table 7.2. The following section will first synthesize the effects of AMF and earthworms on each other. Then the impacts of plant on earthworm performance and AMF activity are discussed before the third part of the section that focuses on the main effects of AMF and earthworms on plant production and plant nutrient content. Finally, a general discussion of the effects of earthworms and AMF on the N:P ratio is engaged followed by an overview of the main interactive effects between AMF, plant and earthworms.

Effects of AMF and earthworms on each other

AMF are known to be a source of food for earthworms (Wolter and Scheu 1999). It has been demonstrated by several authors that earthworms may have a positive effect on the mycorrhization rate by increasing the dispersal of spores (Gange 1993; Reddell and Spain 1991) but also a negative effect by grazing and damaging the external hyphae (Lawrence et al. 2003; Ortiz-Ceballos et al. 2007; Tuffen et al. 2002). Surprisingly, throughout our three experiments, no significant effect of AMF on earthworm biomass or survival has been measured and earthworms did not influence the mycorrhization rate or the hyphal length density of AMF (Table 7.2). The result may be explained as follow. First, it has been demonstrated by Bonkowski et al. (2000) that the preference of five earthworms species for a range of soil fungi follow a general pattern; some fungal species are preferred and other refused. It is consequently possible that in our study *Allolobophora chlorotica* avoided consuming *Glomus intraradices,* which may explain that both species had no influence on each other. Second, this lack of result could be the consequence of opposite effects (i.e. the positive and negative effects mentioned above) that would have cancelled out each other.

Effects of plants on AMF and earthworms' performance

Contrarily to earthworms, the presence of leek roots enhanced the hyphal length density. However, the result must be interpreted cautiously as the AMF inoculum was only introduced in the planted-side of the microcosm and it is probable that the dispersal of external hyphae was reduced by the nylon mesh of 25 µm. The effect of roots on earthworms was less clear; earthworms' survival and biomass was reduced in the two first experiments. It is generally assumed that the soil fauna depends on plant-derived sources entering the belowground system via dead organic material or root exudates. Different earthworm species show thus distinct food preferences for different kind of litter (Curry and Schmidt

Table 7.2 Biological interactions between AMF, earthworms and plant roots. ↑ or ↓: the response variable is significantly increased or decreased ($P < 0.05$) in the presence of the main factors (plant roots, AMF and earthworms). Yes: the response variable is significantly affected ($P < 0.05$) by the explanatory variables, but positive or negative effects cannot be described. ns, not significant.

Response organism	Experiment	Response variable	Root	AMF	Earth-worm	Root x AMF	Root x Worm	AMF x Worm
AMF	1	Mycorrhization rate			ns			
		Hyphal length density	↑		ns		ns	
	2	Mycorrhization rate			ns			
	3	Mycorrhization rate			ns			
Earthworm	1	Total worm number	ns	ns		ns		
		Total worm biomass	↓	ns		ns		
	2	Total worm number	↓	ns		ns		
		Total worm biomass	↓	ns		ns		
	3	Total worm number	ns	ns		ns		
		Total worm biomass	ns	ns		ns		
Plant (biomass)	1	Root biomass		↑	ns			yes
		Shoot biomass		↑	ns			ns
	2	Root biomass		ns	ns			ns
		Shoot biomass		ns	ns			ns
	3	Root biomass		↑	ns			ns
		Shoot biomass		↑	ns			ns
Plant (chemical content)	1	Shoot P concentration		↑	ns			ns
		Shoot N concentration		↑	ns			ns
		N to P ratio		↓	ns			ns
	2	Root P concentration		↑	ns			ns
		Shoot P concentration		↑	↓			ns
		Shoot N concentration		ns	ns			ns
		N to P ratio		↓	↑			ns
	3	Shoot P concentration		ns	↑			ns
		Shoot N concentration		ns	ns			ns
		N to P ratio		ns	ns			ns

2007). Recently, Eisenhauer et al. (2009) demonstrated that earthworms' performance was different according to the quality and quantity of root material of different functional groups as herb, grass and legumes. We therefore suppose that the quality and/or quantity of exudates from leek roots negatively affected earthworms' performance in our experiments. Interestingly, earthworms' performance was however not influenced by leek or petunia roots in the third experiment (Table 7.2). The results may be explained by i) the high mortality rate of earthworms during this third experiment, ii) the experiment duration much shorter than Exp 1 and 2 and iii) the soil used in this last experiment.

Effects of earthworms and AMF on plant performance

During the three experiments, earthworms had no effect on shoot or root biomass, although positive effects were described in previous studies (see Table 1.1 in the first chapter). In Table 1.1, earthworms either influenced positively plant performance or had no significant effect, but the results cannot be attributed to a specific ecological group of earthworms. In particular, Tuffen et al. (2002) showed that *Apporectodea caliginosa*, having an endogeic behavior, significantly improved the biomass of leek shoots and roots. At the opposite, as previously suggested in chapters 2 and 3 (see paragraph above), leek roots are thought to affect negatively the survival of *Allolobophora chlorotica* (Table 7.2). The main cause may be the root exudates that have been suggested to be involved in such a negative effect on earthworm performance. As a consequence, it seems that looking only at the ecological categories of earthworms cannot predict their potential and further effects on plant performance. The general results obtained in the present thesis mixed with other results of previous studies, rather suppose that plant performance is dependent of the close relationships between earthworms and plant species (food preference of earthworms for plant species, root exudation, etc.).

According to previous studies (Table 1.1), the effects of AMF on plant biomass are varying between positive, negative and not significant effects. In Exp 1 and 3, AMF positively affected shoot and root biomass but no significant effect was observed in the second experiment (Table 7.2). Interestingly, the mycorrhization rate was similar (around 70%) between the first and the second experiment. The lack of AMF effect observed in the second experiment could be explained by the mite infection that occurred during the experiment. Herbivores are able to reduce plant growth through loss of plant biomass and photosynthetic area, while plant mutualists, such as AMF, can increase plant growth through uptake of essential nutrients. In our experiment, we did not measure less available P in the soil (see above the section effects on soil chemical properties), and the P content in the shoots and plant biomass were not increased. Assuming mutualism between AMF and the host plant, this result is surprising. However, studies on the tripartite interaction between plant colonized by AMF and herbivores feeding on aboveground parts are scarce, and the effects of AMF on herbivores performance are variable (Hoffmann et al. 2009). Bennett and Bever (2007) suggest that plant response to herbivory depends upon the mycorrhizal fungal mutualist with which a plant is associated. Further investigations are therefore needed to better understand the effects of aboveground herbivory on the AMF-plant symbiosis. Moreover, at the beginning of the experiment, plants seemed to be positively affected by the presence of AMF (visual observation, data not available). Mite infection occurred during the second half of the experiment. Plants had therefore to develop defense mechanisms and AMF become certainly an energetic cost for the plant. Growing without AMF was consequently an advantage for plants that were able to grow at a similar level than plant in symbiosis with AMF; this could explain that AMF did not improve plant performance.

In addition to root and shoot biomass measurements, N and P content have been measured throughout the three experiments. Results of these experiments are summarized in Table 7.2. Overall, and in comparison with previous studies (Table 1.1), no clear

pattern of the effect of earthworm and AMF on N and P in the shoots was highlighted and these effects on P and N content in the shoots cannot predict plant growth (i.e. shoot or root biomass cannot be predicted by an increased P or N content in these parts). Actually, this suggests that other mechanisms like different soil properties, nutrient allocation in the different plant parts (shoot, root, seed, flowers, etc.) that varies with the age of the plants or seasonally may be responsible of such results. Globally, earthworms had no significant effect on P concentration in the shoots in the first experiment, whereas the effect was positive in the second experiment and negative in the third. AMF generally increased P concentration in the shoots and roots, except in the third experiment. By contrast, N concentration in the shoots was only significantly affected by AMF during the first experiment. On the whole, patterns of the effects of AMF on P and N nutrient content in the shoots were similar between our three experiments and studies described in Table 1.1. AMF globally positively affected plant nutrient uptake. Interestingly, except in one case, no significant interactions between AMF and earthworms have been measured during the three experiment of this thesis. Our results support the outcome of Eisenhauer et al. (2009) who suggested that interactions between AMF and earthworms are likely of minor importance.

The N:P ratio, an indicator of soil limiting nutrient

It is widely acknowledged that plant growth strongly depends on limiting nutrients as N and P in the soil. The N:P ratio has been shown to be a good indicator to detect the nature of nutrient limitation (Koerselman and Meuleman 1996). According to Güsewell (2004), the production of vegetative biomass is enhanced by N fertilization with a N:P ratio < 10 (N is limiting) and by P fertilization with a N:P ratio > 20 (P is limiting). Intermediary ratios are supposed to reflect that either N or P is limiting or that plant growth is co-limited by N and P (Koerselman and Meuleman 1996). The N:P ratio is therefore a very interesting tool to assess by measuring total N and P content in the shoots which soil nutrient is limiting. As demonstrated in the first section of this chapter, AMF and earthworms are able to modify the soil chemical properties and particularly the N and P content in the soil. They are consequently able to affect the N:P ratio. We therefore hypothesize that in P limiting conditions (N:P ratio > 20), AMF would be key organisms by improving plant P uptake, whereas in N limited condition, earthworms would play a major role by enhancing N mineralization. At intermediary ratio, AMF and/or earthworms are supposed to influence the N:P ratio.

In the first experiment (Fig. 7.1 a), the N:P ratio of plants grown without AMF was greater than 20. According to Gusewell (2004), P is limiting and an increased P uptake is required for enhancing the plant biomass. The presence of AMF significantly reduced the N:P ratio. This result confirms therefore that AMF beneficially influenced plant growth by improving the uptake of P by plants without external addition of P. In the second experiment, (Fig. 7.1 b), the N:P ratio of plants grown without P addition was comprised between 10 and 20, which suggests that fertilization with N, P or both nutrients may improve the plant biomass. In this case of an intermediary N:P ratio, both AMF and earthworms significantly but contrarily affected the N:P ratio (Table 7.2). This is a very interesting result that is in accordance with our hypothesis. Indeed, it is not surprising that both soil organisms have a significant effect in the

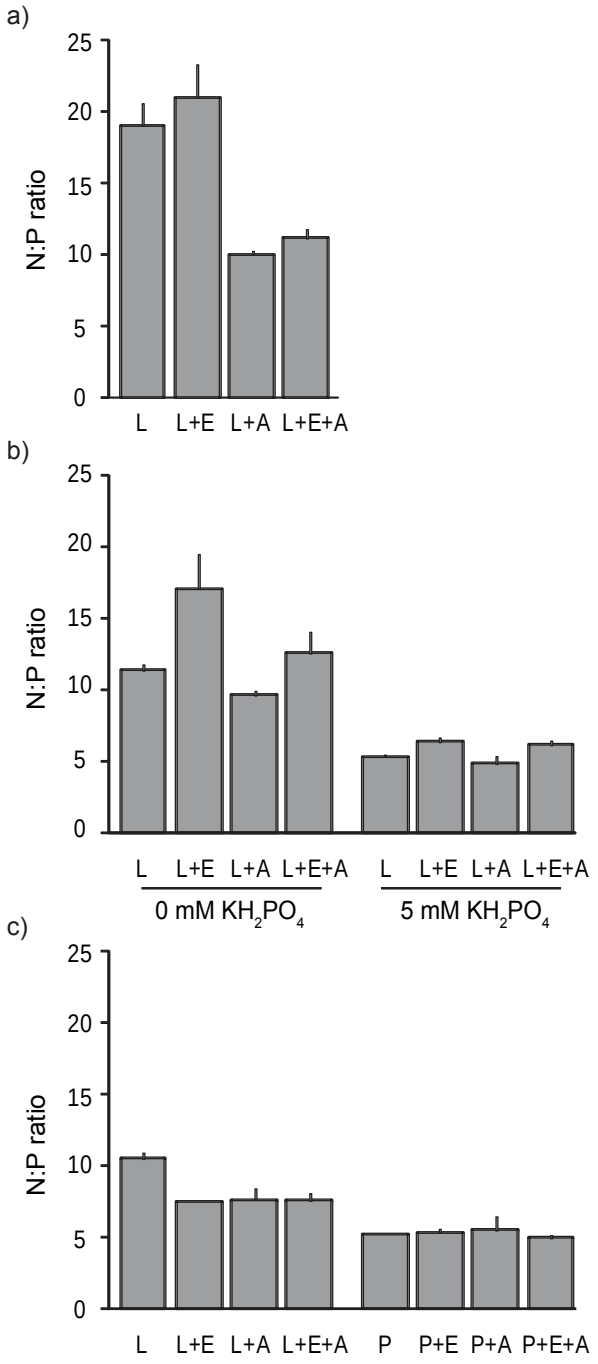

Fig. 7.1 Effect of the presence of earthworms (E), AMF (A), leek (L) or petunia (P) on the N:P ratio as measured a) in the first experiment in climate chamber after 35 weeks, b) in the second experiment in glasshouse after 45 weeks with or without P fertilization, and c) in the third experiment in glasshouse after 22 weeks. The soil used is a loamy Anthrosol for the two first experiments and a silt loam Luvisol in the third experiment.

case of a middle N:P ratio. In addition, it seems important to highlight that the N:P ratio is lower in the second experiment compared with the first experiment using the same soil. It has been demonstrated that N:P ratios depend on several factors. Among these factors, N:P ratios decrease with plant age (Gusewell 2004). The second experiment lasted 10 weeks more than the first one, which may explain the lower N:P ratio. Finally, the N:P ratios of plants grown during the third experiment were very low, thus indicating a strong N limitation (Fig. 7.1 c). This small ratio may be explained by the available P content in the soil of the third experiment that was two times higher than in the two first experiments. In this case, the N:P ratio was not significantly affected by AMF nor earthworms. According to our hypotheses, earthworms were supposed to have enhanced the mineralization of nitrogen and have significantly increased the N:P ratio. As this was not the case, we therefore suppose that the result is due to biological effects like the species or the ecological category of earthworm, or to a temporal effect. In addition, this third experiment lasted 22 weeks, which can be too short for earthworms to significantly influence the N:P ratio. Further experiments aiming at better understanding the role of soil fauna on the N:P ratio would be interesting.

Overview of the interacting effects of AMF, plants and earthworms

Overall, several biological interactions have occurred between soil organisms and some general patterns about the effect of a species on another may be highlighted:

i. *Allolobophora chlorotica* and *Glomus intraradices* have no effect on each other.

ii. *Allolobophora chlorotica* has no influence on the biomass of *Allium porrum* or *Petunia*

hybrida.

iii. *Allium porrum* generally negatively affects *Allolobophora chlorotica* performance (weight and/or survival).

iv. *Glomus intraradices* and *Allium porrum* or *Petunia hybrida* beneficially affect each other.

v. *Glomus intraradices* and *Allolobophora chlorotica* have generally no interactive effects on plant performance.

vi. No clear pattern of the effects of *Glomus intraradices* and *Allolobophora chlorotica* on N or P content in the shoots has been highlighted.

Perspectives

This section will give some general perspectives and idea for future experiments. However, some general considerations are first presented. Beyond the effects of AMF, plants, and earthworms on soil physical, chemical and biological properties, this thesis allowed to highlight the importance of studying the interaction of organisms of different functional groups to better understand the synergistic or antagonistic effects that may occur. In addition, results of the thesis point out the importance of the initial experimental conditions and the temporal variations in this kind of experiments. Differences between studies are probably related to the initial factors like microcosm size, soil properties, experiment location (climate chamber *vs.* glasshouse), temperature, moisture, etc. or to the length of experiments that differ greatly among studies.

The microcosm design employed in the present thesis is a common widely used technique for testing the role of soil biota in the soil ecosystem. Indeed, microcosm systems are considered as a representative model of the field. Microcosm studies have been employed to test for the effect of different assemblage of soil biota on soil decomposition or mineralization processes (see for example Huhta 2007). In this thesis, microcosms consisted in cylindrical pots filled with soil in which three soil organisms were introduced individually or in combination. Although the system is very simple, biotic interactions among organisms and the effects of each organism on soil properties become rapidly highly complex, especially because individual effects of soil organism in combination may cancel out each other. A lot of studies have already assessed the effect of AMF on plant communities or the effect of a combination of different AMF species on plant communities (Klironomos 2003; van der Heijden et al. 1998a). A similar experiment combining the effect of a mixture of earthworm species on plant communities is to my knowledge still lacking, as well as the effect of the combination of different ecological categories of earthworms with different AMF species. Future studies should therefore focus on the effect of different earthworms and AMF communities rather than working on a single species independently. Moreover, results of the thesis highlighted the key role of the soil types and the duration of the experiments. We showed that the effects of soil organisms were different according to the nature of the soil in which they were introduced. However, the two soils used in our experiments were employed in different experiments and a comparison is consequently difficult. Future studies should therefore better take into account the whole soil properties by testing different soil types in a single experiment.

Results of shrinkage analyses (see chapter 4 and 5) are promising. To our knowledge, it is the first report of such an approach where shrinkage analysis is successfully applied to assess the physical impact of soil biota in the soil. In particular, we demonstrated that different soil organisms (earthworms, plant roots and AMF) have different effects on the multiple soil properties measured with shrinkage analysis. More interestingly, the thesis highlighted the essential role of soils having different initial properties in influencing the effect of soil biota. An experiment aiming at better understanding the effect of different earthworm ecological categories with two different soils at different compaction rate is already in progress. Moreover, future experiments should also take into account and test the role of roots (e.g root exudates) or external hyphae (e.g. glomalin) on the soil structure and more particularly on shrinkage analyses and the pore size distribution.

The mite infection that occurred during the second glasshouse experiment highlighted the importance of the aboveground system. It is evident that soil organisms, organized in primary, secondary or higher consumers in the soil food web, strongly depend of the quality and quantity of resource produced by the primary producers. However, the above part of plants (i.e. the shoots) may also be affected by primary consumers as shoot herbivores. The latter may consequently influence the quantity or quality of litter that return to the soil (Wardle 2002). Shoot herbivores may also strongly affect the carbon allocation (photosynthates) of the plants to the roots and consequently modify root exudates and the remaining soil food web (Hooper et al. 2000). This may be particularly important for the AMF-root symbiosis (Van der Putten et al. 2001). It would be therefore interesting to design experiments integrating both the above- and belowground system by adding shoot pathogens or herbivores in the experiments. Indeed, recent studies show promising result of such approaches (Engelkes et al. 2008; Erb et al. 2008; Hladun and Adler 2009).

Finally, with the increasing number of factors interesting or suggesting for future experiments, it becomes more and more difficult to perform and control experimental designs using microcosms. Two complementary approaches may therefore be helpful for a better understanding of the mechanisms involved in the functioning of complex soil ecosystem processes. First, as a complement to microcosm studies, field experiments are necessary, either by modifying the soil communities in place or using a controlled introduction of new species in the field (Baker et al. 2006; Lawrence et al. 2003; Van der Heijden et al. 1998b). Second, as biological and physico-chemical interactions are complex and because sampling is difficult, dynamic simulation models may be a useful tool for overcoming these numerous constraints. It may also allow generating new hypotheses that can be in turn evaluated empirically. However, modelling the soil environment is a challenge because the soil is a multi-scale heterogeneous, three-dimensional and dynamic environment. Modelling the effects of soil invertebrates on soil aggregation and porosity (Blanchart et al. 2009; Marilleau et al. 2008) or the effect of AMF or roots on nutrient uptake (Dunbabin et al. 2002; Pierret et al. 2007; Schnepf et al. 2008) are still in progress but as suggested by Roose and Schnepf (2008), future prospects in dynamical modelling of plant-soil-invertebrates are encouraged.

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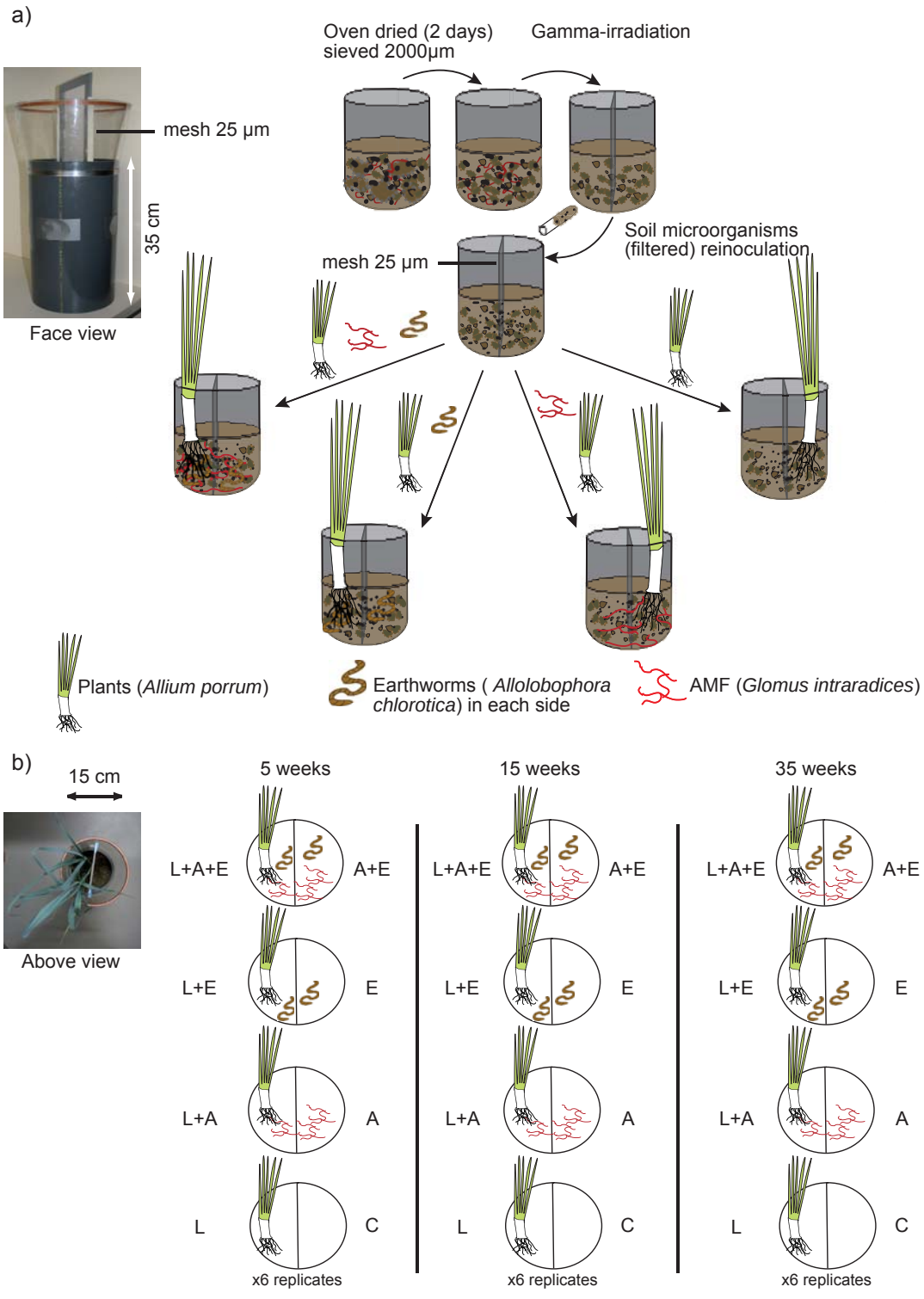
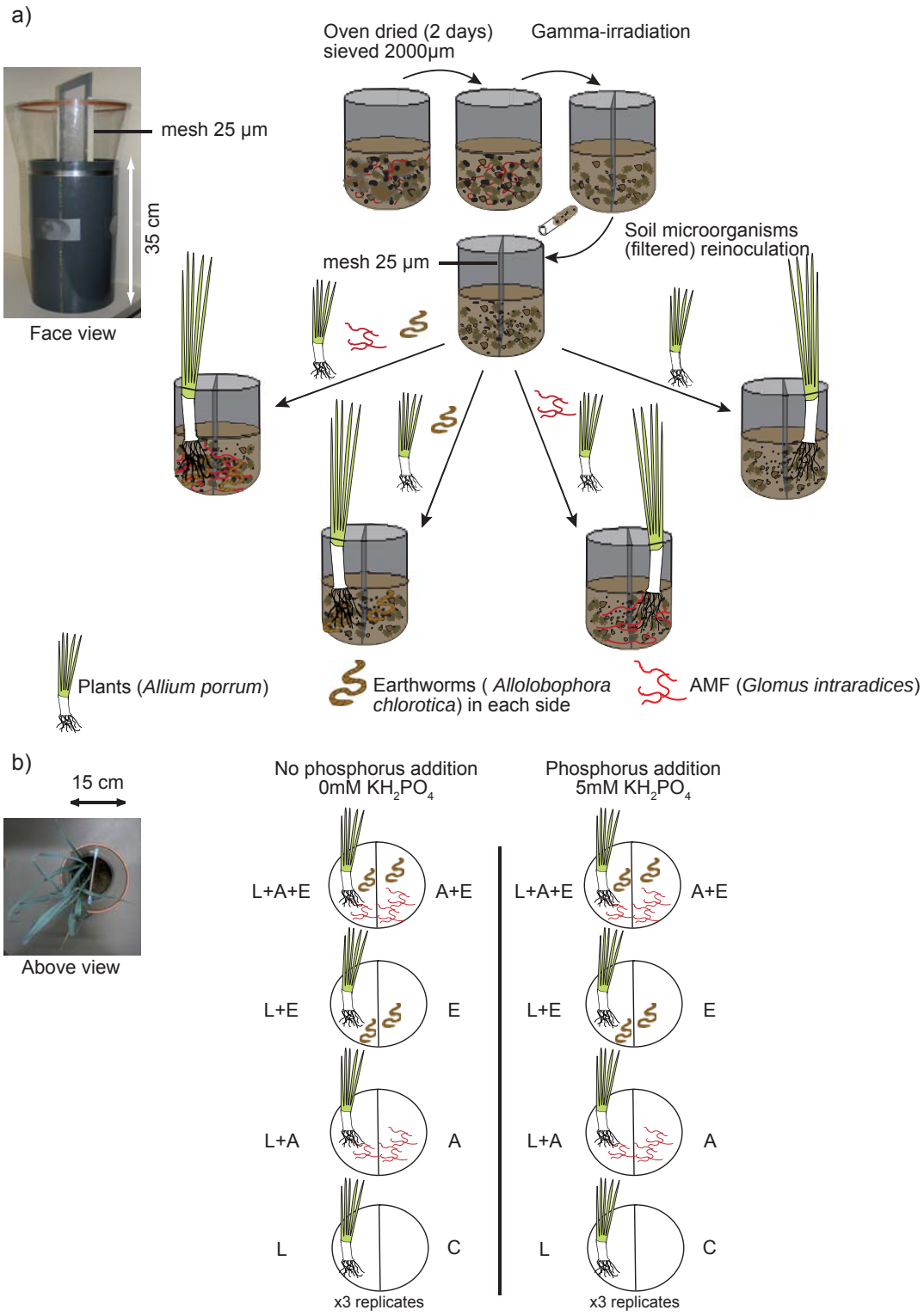
Appendices

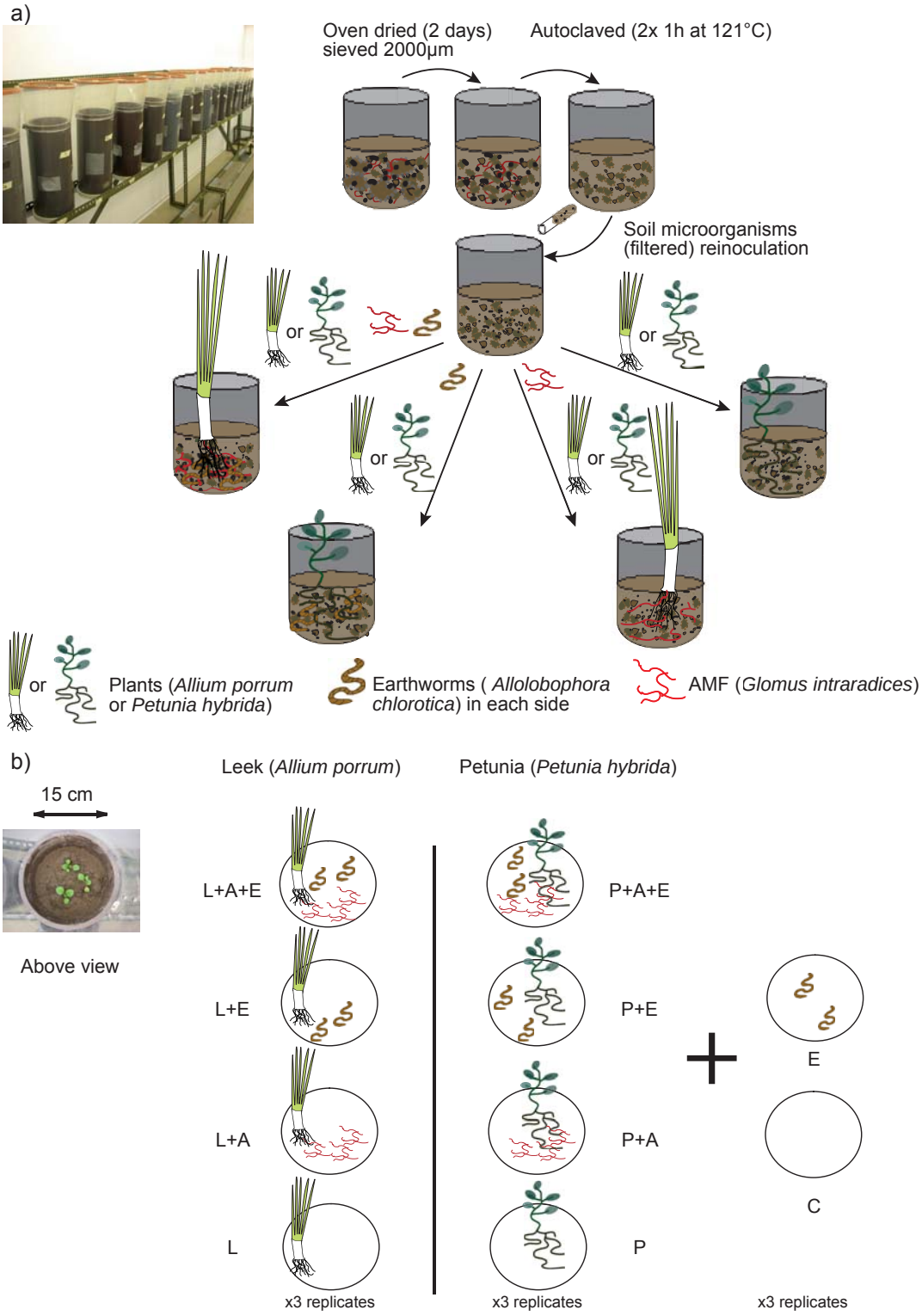
Appendix 1 Experimental design of the first experiment (a). Each microcosm received 4,5 kg of air-dried, sieved (2 mm) soil (loamy Anthrosol) that was gamma-irradiated and re-inoculated with a filtered washings of initial soil before receiving three leek plantlets in one side. AMF and earthworms were then randomly introduced into the microcosms. (b) Above view of the experimental design. Eight treatments (two treatments per microcosm) were obtained, according to the presence/absence of plants, earthworms and AMF. The experiment was conducted in a climate chamber (photoperiod 16/8 h (day(night), temperature 18±2°C, 50% humidity). Leek plant (L), AMF (A), earthworm (E), and control (C). Each microcosm was replicated 6 times for each harvest time: 5 weeks, 15 weeks and 35 weeks.



Appendix 2 Experimental design of the second experiment (a). Each microcosm received 4,5 kg of air-dried, sieved (2 mm) soil (loamy Anthrosol) that was gamma-irradiated and re-inoculated with a filtered washings of initial soil before receiving three leek plantlets in one side. AMF and earthworms were then randomly introduced into the microcosms. (b) Above view of the experimental design. Eight treatments (two treatments per microcosm) were obtained, according to the presence/absence of plants, earthworms and AMF and P fertilization (0 mM KH_2PO_4 or 5 mM KH_2PO_4). The experiment lasted 45 weeks in a glasshouse (Botanical garden, Neuchâtel). Leek plant (L), AMF (A), earthworm (E), and control (C). Each microcosm was replicated three times.



Appendix 3 Experimental design of the third experiment (a). Each microcosm received 4,5 kg of air-dried, sieved (2 mm) soil (silt loam Luvisol) that was autoclaved (2 times, 1 h at 121 °C) and re-inoculated with a filtered washings of initial soil. Three leek plantlets, AMF and earthworms were then randomly introduced into the microcosms. (b) Above view of the experimental design. Ten treatments were obtained according to the presence/absence of plants, earthworms and AMF. The experiment lasted 22 weeks under glasshouse (HEPIA, Jussy). Leek plant (L), petunia plants (P), AMF (A), earthworm (E), and control (C). Each microcosm was replicated 3 times.



Appendix 4: List of Tables

Appendix 5: List of Figures

Appendix 6: Curriculum Vitae

Roxane Kohler-Milleret
Born: 30th of November 1979, Chêne-Bougeries (GE), Switzerland
Nationality: Swiss, French
Marital status: married
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Education

2005 - 2009 **Ph.D.** Institute of Biology, Laboratory Soil & Vegetation, University of Neuchâtel, Switzerland.
Ph.D thesis: Roots, earthworms and mycorrhizae: Individual and interactive effects on some chemical, physical and biological properties of soils

1999 – 2004 **B.Sc. and M.Sc**. Department of Ecology and Evolution, University of Lausanne, Switzerland.
Master thesis: Impact of climate change on the distribution of plant species

Professional career

2005 – 2009 **Teaching Assistant,** Institute of Biology, Laboratory Soil & Vegetation, University of Neuchâtel, Switzerland.

2003 – 2004 **Teaching Assistant,** Department of Ecology and Evolution, University of Lausanne, Switzerland

List of publication in peer-reviewed journal

Roxane Milleret, Renée Claire Le Bayon, Frédéric Lamy, Jean-Michel Gobat, Pascal Boivin (2009) Impact of roots, mycorrhizas and earthworms on soil physical properties as assessed by shrinkage analysis. Journal of Hydrology (373), 499-507.

Laure Weisskopf, Pollycarp Akello, **Roxane Milleret**, Zeyaur R. Khan, Fritz Schulthess, Jean-Michel Gobat, Renée Claire Le Bayon (2009) White lupin leads to increased maize yield through a soil fertility-independent mechanism: a new candidate for fighting *Striga hermontica* infestation? Plant and soil (319), 101-114.

Roxane Milleret, Renée Claire Le Bayon, Jean-Michel Gobat (2009) Root, mycorrhiza and earthworm interactions: their effects on soil structuring processes, plant and soil nutrient concentration and plant biomass. Plant and soil (316), 1-12.

Roxane Milleret, Sonia Tarnawski, Renée Claire Le Bayon, Jean-Michel Gobat The influence of plant roots, earthworms and mycorrhizae on genetic and functional structures of bacterial communities. (*submitted*).

Renée Claire Le Bayon, **Roxane Milleret** Effects of earthworms on phosphorus dynamics - a review. (*submitted*)

CONFERENCE PROCEEDINGS & INTERNATIONAL MEETINGS

ROXANE MILLERET, RENÉE CLAIRE LE BAYON, JEAN-MICHEL GOBAT Earthworm, arbuscular mycorrhizal fungi (AMF) and root interactions: their effects on soil fertility as described by soil nutrient status, bacterial community structure and soil stability. Société Suisse de Pédologie 2009, 5 - 6 janvier 2009, Wädenswil, Suisse (Oral communication).

FRANÇOIS FÜLLEMANN, **ROXANE MILLERET**, PASCAL BOIVIN Evaluation de l'influence des catégories écologiques de lombriciens sur les propriétés structurales du sol. Société Suisse de Pédologie 2009, 5 - 6 janvier 2009, Wädenswil, Suisse (Poster).

ROXANE MILLERET, SONIA TARNAWSKI, RENÉE CLAIRE LE BAYON, JEAN-MICHEL GOBAT How different soil organisms affect total and functional bacterial communities structures? Swiss Microbial Ecology 2009, 28 - 30 janvier 2009, Einsiedeln, Suisse (Poster).

ROXANE MILLERET, RENÉE CLAIRE LE BAYON, JEAN-MICHEL GOBAT Root, mycorrhiza, and earthworm interactions: their combined effects on plant biomass, nutrition and soil structure. Eurosoil 2008, 25 - 29 août 2008, Vienne, Autriche (Oral communication).

PASCAL BOIVIN, **ROXANE MILLERET**, RENÉE CLAIRE LE BAYON, JEAN-MICHEL GOBAT, FRÉDÉRIC LAMY Impact of root growth, fungi and earthworms on soil physical properties as assessed by shrinkage analysis. Eurosoil 2008, 25 - 29 août 2008, Vienne, Autriche (Oral communication).

ROXANE MILLERET, SONIA TARNAWSKI, RENÉE CLAIRE. LE BAYON, JEAN-MICHEL GOBAT Influence of earthworms, mycorrhiza and plant roots on soil bacterial metabolic community pattern. Eurosoil 2008, 25 - 29 août 2008, Vienne, Autriche (Poster).

ROXANE MILLERET, RENÉE CLAIRE LE BAYON, JEAN-MICHEL GOBAT Interactions between plant, AMF and earthworms: a mesocosm experiment. IIIe Cycle de Biologie Végétale Molecular Genetics of Root Endosymbioses, 6 juin 2007, Cartigny, Suisse (Poster).

ROXANE MILLERET, RENÉE CLAIRE LE BAYON, JEAN-MICHEL GOBAT Combined effects of earthworms and arbuscular mycorrhizal fungi (AMF) on soil structure processes : A microcosm study. The 8th International Symposium on Earthworm ecology, 4 - 9 Septembre 2006, Cracovie, Pologne (Poster).

ROXANE MILLERET, CHRISTOPHE RANDIN, ROBIN ENGLER, ANTOINE GUISAN What will be the fate of plant species in a climate warming scenario by 2100? A spatial simulation study. 5th Swiss Global Change Day, Bern, 5 avril 2004 (Poster).