Plant Soil (2009) 316:1–12
DOI 10.1007/s11104-008-9753-7

REGULAR ARTICLE

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

Roxane Milleret · Renée-Claire Le Bayon · Jean-Michel Gobat

Received: 25 June 2008 / Accepted: 11 August 2008 / Published online: 4 September 2008

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

Responsible Editor: SE Hans Lambers.

R. Milleret (✉) · R.-C. Le Bayon · J.-M. Gobat
Institute of Biology, University of Neuchâtel,
Laboratory Soil and Vegetation,
Emile Argand 11, CP 158,
2009 Neuchâtel, Switzerland
e-mail: roxane.milleret@unine.ch

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 and 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 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 1,000 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 per square metre 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 3 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 250× 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 min, 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, 4×1 ml aliquots (10 s 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 ($WSA_{1-2\ mm}$) 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 min and wet-sieved 10 min 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) × 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×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 ($WSA_{1–2\ mm}$) (Table 1). With earthworms, the percentage of $WSA_{1–2\ mm}$ was significantly lower (25.8±1.0%) than without earthworms (31.6±1.1%) (Fig. 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×t, L×t and L×A were also significant. AMF and leek together enhanced the percentage of water stable macroaggregates compared to leek alone (Fig. 2a).

Chemical analyses

AMF, leek and time significantly affected the amount of available P (P_a) in the bulk soil (Table 1). Available P in the bulk soil was significantly lower when AMF and leek were added (Fig. 3a). The L×A interaction was also significant. Available P was lower when both AMF and leek were present in the microcosm (Fig. 2b). Moreover, the interactions A×t and L×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 (Fig. 3b). However, the E×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. 2c). Total C showed similar pattern than total N (Figs. 2d and 3c). Moreover, total C in the BS was significantly affected by time and the interactions E×t and A×t (Table 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). Drilosphere soil contained more P_a and total N and less total C compared to rhizosphere and bulk soil (Fig. 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. 4a). No effect of AMF was observed on total C and N (Fig. 4b,c).

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

Total fresh root biomass varied significantly with AMF and time (Table 3). The interactions A×t and E×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. 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

Table 1 Partly nested ANOVA showing the effect of earthworms, AMF, leek and time on the percentage of water stable macroaggregates ($WSA_{1–2\ mm}$), available P (μg g^{-1}) and total carbon and nitrogen content (mg g^{-1}) in the bulk soil

Bulk soil analysis	*df*	Physical parameters		Chemical parameters					
		$WSA_{1–2\ mm}$		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×*A*	1	2.69	ns	0.63	ns	9.1	**	8.72	**
E×*t*	2	11.88	***	1.61	ns	11.18	***	1.63	ns
A×*t*	2	0.47	ns	10.08	***	5.55	**	1.47	ns
Residuals (MS)	62	47.75		6.76		0.08		1.14×10^{-3}	
Within microcosms									
L	1	28.49	***	40.78	***	1.65	ns	0.76	ns
E×*L*	1	1.07	ns	0.85	ns	1.08	ns	0.04	ns
A×*L*	1	4.64	*	6.69	*	1.25	ns	1.34	ns
L×*t*	2	30.91	***	24.44	***	1.56	ns	1.18	ns
Residuals (MS)	67	32.70		6.84		0.06		0.30×10^{-3}	

E Earthworms, *A* AMF, *L* leek plants, *t* time of harvest, *df* degrees of freedom, *MS* mean square, *ns* not significant
*$P<0.05$, **$P<0.01$, ***$P<0.001$

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 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,

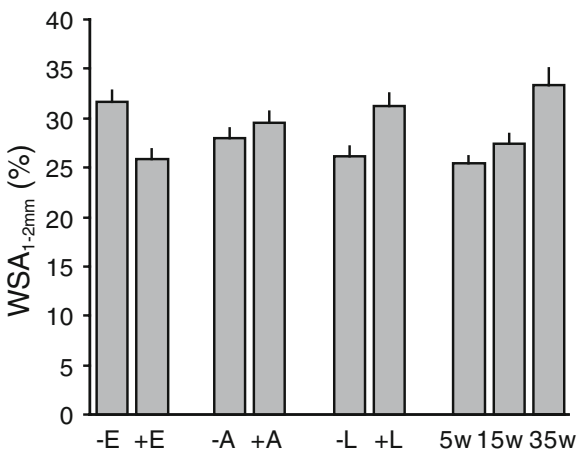

Fig. 1 Main effects of the presence of earthworms (E, *Allolobophora chlorotica*), AMF (A, *Glomus intraradices*), leek (L, *Allium porrum*) and time of harvest with complete destruction of microcosms after 5, 15 and 35 weeks (*5 w*, *15 w*, *35 w*) on the percentage of water-stable macroaggregates in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

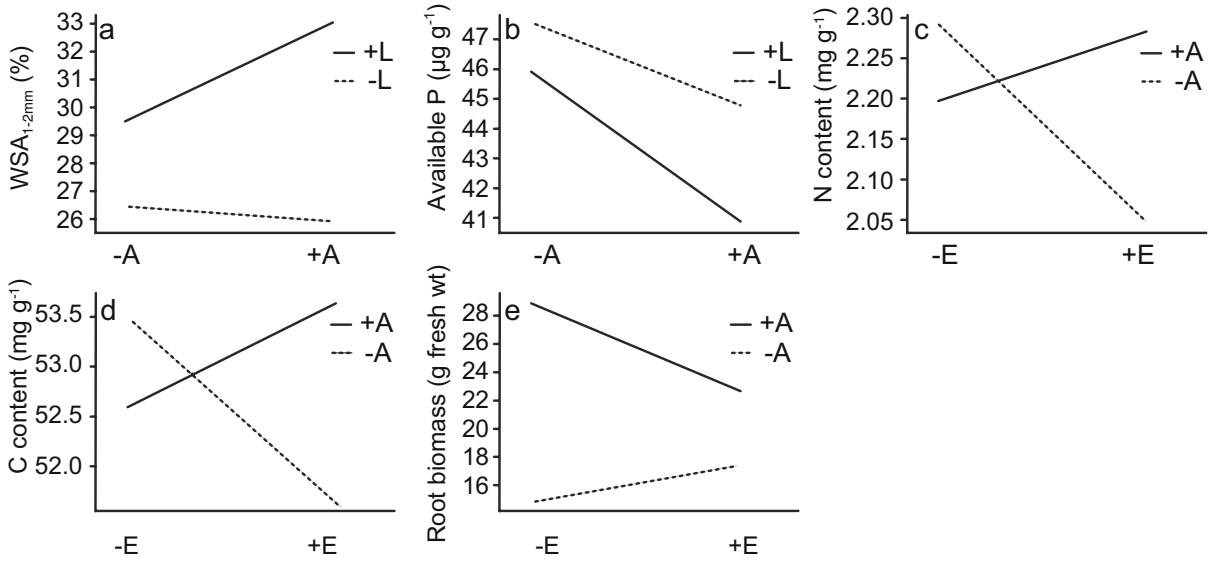

Fig. 2 Plot of the significant interactions of AMF (A, *Glomus intraradices*) and leek (L, *Allium porrum*) on **a** the percentage of water stable macroaggregates in the 1–2 mm size class ($WSA_{1-2\ mm}$) and **b** available P in the bulk soil and the significant interactions between AMF (A, *Glomus 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)

but become more stable after aging and drying (Shipitalo and Protz 1989). In our long-term experiment (35 weeks), earthworms occupied almost all the 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.

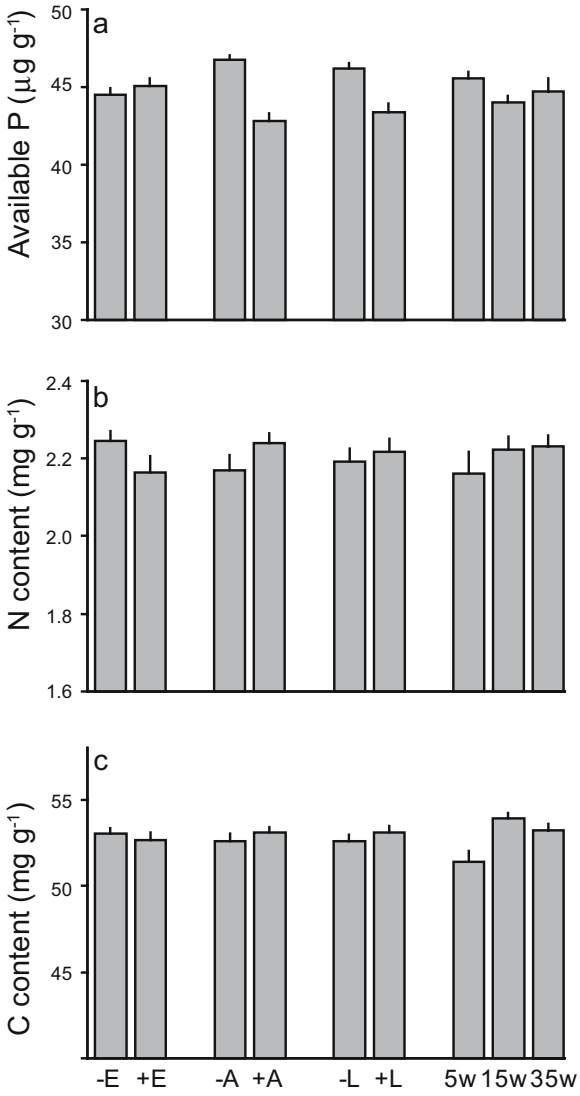

Fig. 3 Main effects of earthworms (E, *Allolobophora chlorotica*), AMF (A, *Glomus intraradices*), leek (L, *Allium porrum*) and time of harvest with complete destruction of microcosms after 5, 15 and 35 weeks (*5 w*, *15 w*, *35 w*) on **a** available P (µg g^{-1}), **b** total N content (mg g^{-1}) in the bulk soil and **c** total C content (mg g^{-1}) in the bulk soil. Bar represents mean±SE

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

Table 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})

	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×*t*	1	7.35	*	0.92	ns	0.17	ns
Residuals (MS)	20	17.62		0.02		0.53×10^{-3}	
Within microcosms							
sc	2	10.38	***	16.11	***	4.17	*
sc×*A*	2	0.59	ns	0.41	ns	1.87	ns
sc×*t*	2	6.58	**	1.70	ns	0.72	ns
Residuals (MS)	42	9.26		0.05		0.56×10^{-3}	

A AMF, *t* time of harvest, *sc* soil compartment (bulk soil, rhizosphere, or drilosphere soil), *df* degrees of freedom, *MS* mean square, *ns* not significant

*$P<0.05$, **$P<0.01$, ***$P<0.001$

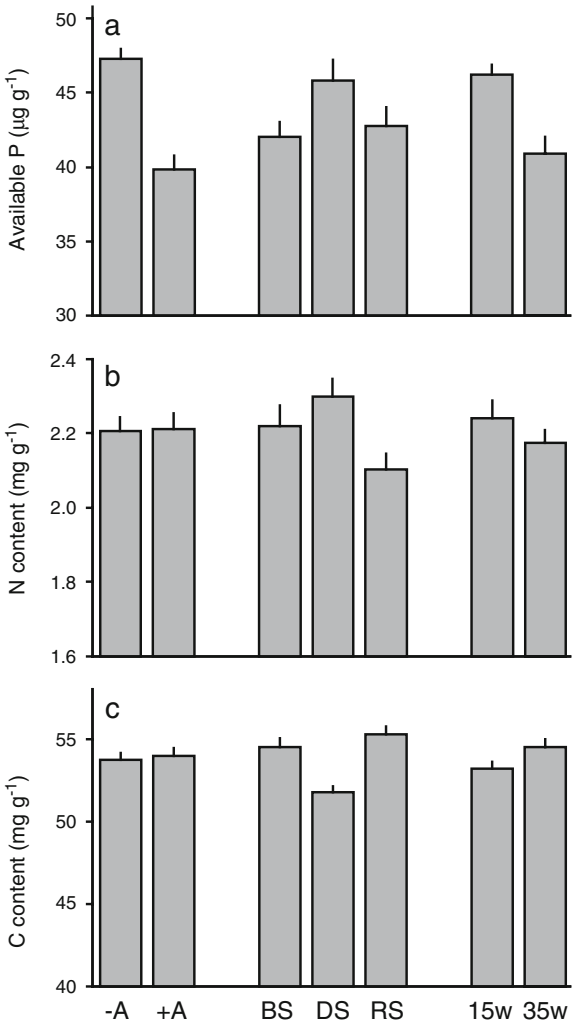

Fig. 4 Main effects of AMF (A, *Glomus intraradices*), soil compartment (*BS* bulk soil, *DS* drilosphere soil, *RS* rhizosphere soil), and time of harvest with complete destruction of microcosms after 15 and 35 weeks (*15 w*, *35 w*) on **a** available P (µg g^{-1}), **b** total nitrogen content (mg g^{-1}) and **c** total carbon content (mg g^{-1}). DS and RS samples after 5 weeks were not available. Bar represents mean±SE

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

Table 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 and fresh root weight (g) after each time of harvest (5, 15 or 35 weeks)

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×*A*			0.01	0.37	0.75	0.74	0.61
E×*t*			0.25	0.06	0.93	0.59	0.65
A×*t*			<0.001	<0.001	0.27	0.09	0.16

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

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 pros-

pects should therefore better take into account chemical and physical soil parameters.

Acknowledgments This project was funded by the National Centre of Competence in Research (NCCR) Plant Survival, a research programme of the Swiss National Science Foundation. The authors are very grateful to Dr Jan Jansa for his help in hyphal length density determination. We also thank Lidia Mathys-Paganuzzi and Marie-Laure Heusler for their excellent technical assistance as well as the botanical garden of Neuchâtel. We also gratefully thank Drs Florian Kohler and François Gillet for their help in statistical analyses.

References

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ (1998) Soil aggregation status and rhizobacteria in the mycorrhizosphere. Plant Soil 202:89–96 doi:10.1023/A:1004301423150

Angers DA, Caron J (1998) Plant-induced changes in soil structure: processes and feedbacks. Biogeochemistry 42:55–72 doi:10.1023/A:1005944025343

Blanchart E, Albrecht A, Brown G, Decaens T, Duboisset A, Lavelle P et al (2004) Effects of tropical endogeic earthworms on soil erosion. Agric Ecosyst Environ 104:303–315 doi:10.1016/j.agee.2004.01.031

Boivin P, Schaffer B, Temgoua E, Gratier M, Steinman G (2006) Assessment of soil compaction using modelling: Experimental data and perspectives. Soil Tillage Res 88:65–79 doi:10.1016/j.still.2005.04.008

Brown GG, Edwards CA, Brussaard L (2004) How earthworms affect plant growth: burrowing into the mechanisms. In: Edwards CA (ed) Earthworm ecology. CRC, Boca Raton, USA, pp 13–49

Coleman DC (2008) From peds to paradoxes: linkage between soil biota and their influences on ecological processes. Soil Biol Biochem 40:271–289 doi:10.1016/j.soilbio.2007.08.005

Decaens T, Rangel AF, Asakawa N, Thomas RJ (1999) Carbon and nitrogen dynamics in ageing earthworm casts in grasslands of the eastern plains of Colombia. Biol Fertil Soils 30:20–28 doi:10.1007/s003740050582

Devleeschauwer D, Lal R (1981) Properties of worm casts under secondary tropical forest regrowth. Soil Sci 132:175–181

Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms. Chapman & Hall, London, UK, 426 p

Endlweber K, Scheu S (2007) Interactions between mycorrhizal fungi and Collembola: effects on root structure of competing plant species. Biol Fertil Soils 43:741–749 doi:10.1007/s00374-006-0157-7

Guggenberger G, Thomas RJ, Zech W (1996) Soil organic matter within earthworm casts of an anecic-endogeic tropical pasture community, Colombia. Appl Soil Ecol 3:263–274 doi:10.1016/0929-1393(95)00081-X

Hawkins HJ, Johansen A, George E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226:275–285 doi:10.1023/A:1026500810385

ISSS Working group RB (1998) World reference base for soil resources: introduction. Acco, Leuven

Jakobsen I, Abbott LK, Robson AD (1992) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with trifolium-subterraneum L. 1. Spread of hyphae and phosphorus inflow into roots. New Phytol 120:371–380 doi:10.1111/j.1469-8137.1992.tb01077.x

Jansa J, Mozafar A, Frossard E (2005) Phosphorus acquisition strategies within arbuscular mycorrhizal fungal community of a single field site. Plant Soil 276:163–176 doi:10.1007/s11104-005-4274-0

Kemper WD, Rosenau RC (1986) Aggregate stability and size distribution. In: Klute A (ed) Methods of soil analysis, part 1. Physical and mineralogical methods. Soil Science Society of America, Madison, USA, pp 425–442 (book series, 5)

Koerselman W, Meuleman AFM (1996) The vegetation N:P ratio: A new tool to detect the nature of nutrient limitation. J Appl Ecol 33:1441–1450 doi:10.2307/2404783

Koide RT, Li MG (1989) Appropriate controls for vesicular arbuscular mycorrhiza research. New Phytol 111:35–44 doi:10.1111/j.1469-8137.1989.tb04215.x

Lawrence AP, Bowers MA (2002) A test of the 'hot' mustard extraction method of sampling earthworms. Soil Biol Biochem 34:549–552 doi:10.1016/S0038-0717(01)00211-5

Le Bayon RC, Binet F (1999) Rainfall effects on erosion of earthworm casts and phosphorus transfers by water runoff. Biol Fertil Soils 30:7–13 doi:10.1007/s003740050580

Le Bayon RC, Binet F (2006) Earthworms change the distribution and availability of phosphorous in organic substrates. Soil Biol Biochem 38:235–246

Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant Soil 159:89–102

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective-measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. New Phytol 115:495–501 doi:10.1111/j.1469-8137.1990.tb00476.x

McNamara NP, Black HIJ, Beresford NA, Parekh NR (2003) Effects of acute gamma irradiation on chemical, physical and biological properties of soils. Appl Soil Ecol 24:117–132 doi:10.1016/S0929-1393(03)00073-8

Murphy J, Riley JP (1962) A modified single solution method for determination of phosphate in natural waters. Anal Chim Acta 27:31–36 doi:10.1016/S0003-2670(00)88444-5

Newman EI (1966) A method of estimating total length of root in a sample. J Appl Ecol 3:139–145 doi:10.2307/2401670

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of available phosphorous in soils by extraction with sodium bicarbonate. USDA Circular 939:1–8

Ortiz-Ceballos AI, Pena-Cabriales JJ, Fragoso C, Brown GG (2007) Mycorrhizal colonization and nitrogen uptake by maize: combined effect of tropical earthworms and velvetbean mulch. Biol Fertil Soils 44:181–186 doi:10.1007/s00374-007-0193-y

Piotrowski JS, Denich T, Klironomos JN, Graham JM, Rillig MC (2004) The effects of arbuscular mycorrhizas on soil aggregation depend on the interaction between plant and fungal species. New Phytol 164:365–373 doi:10.1111/j.1469-8137.2004.01181.x

R Development Core Team (2007) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure. New Phytol 171:41–53 doi:10.1111/j.1469-8137.2006.01750.x

Rillig MC, Wright SF, Eviner VT (2002) The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. Plant Soil 238:325–333 doi:10.1023/A:1014483303813

Scheu S (1994) There is an earthworm mobilizable nitrogen pool in soil. Pedobiologia (Jena) 38:243–249

Schreiner RP, Mihara KL, McDaniel H, Bethlenfalvay GJ (1997) Mycorrhizal fungi influence plant and soil functions and interactions. Plant Soil 188:199–209 doi:10.1023/A:1004271525014

Shipitalo MJ, Protz R (1989) Chemistry and micromorphology of aggregation in earthworm casts. Geoderma 45:357–374 doi:10.1016/0016-7061(89)90016-5

Six J, Feller C, Denef K, Ogle SM, Sa JCD, Albrecht A (2002) Soil organic matter, biota and aggregation in temperate and tropical soils – effects of no-tillage. Agronomie 22:755–775 doi:10.1051/agro:2002043

Six J, Bossuyt H, Degryze S, Denef K (2004) A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil Tillage Res 79:7–31 doi:10.1016/j.still.2004.03.008

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, London, UK, 605 p

Smith SE, Smith FA, Jakobsen I (2004) Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytol 162:511–524 doi:10.1111/j.1469-8137.2004.01039.x

Sorensen JN, Larsen J, Jakobsen I (2005) Mycorrhiza formation and nutrient concentration in leeks (Allium porrum) in relation to previous crop and cover crop management on high P soils. Plant Soil 273:101–114 doi:10.1007/s11104-004-6960-8

Sorensen JN, Larsen J, Jakibsen I (2008) Pre-inoculation with arbuscular mycorrhizal fungi increases early nutrient concentration and growth of field-grown leeks under high productivity conditions. Plant Soil 307:135–147 doi:10.1007/s11104-008-9591-7

Tisdall JM, Oades JM (1982) Organic-matter and water-stable aggregates in soils. J Soil Sci 33:141–163 doi:10.1111/j.1365-2389.1982.tb01755.x

Tuffen F, Eason WR, Scullion J (2002) The effect of earthworms and arbuscular mycorrhizal fungi on growth of and P-32 transfer between Allium porrum plants. Soil Biol Biochem 34:1027–1036 doi:10.1016/S0038-0717(02)00036-6

van der Heijden MGA, Boller T, Wiemken A, Sanders IR (1998) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082–2091

van der Heijden MGA, Streitwolf-Engel R, Riedl R, Siegrist S, Neudecker A, Ineichen K et al (2006) The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytol 172:739–752 doi:10.1111/j.1469-8137.2006.01862.x

Wardle DA (2002) Communities and ecosystems: linking the aboveground and belowground components. Princeton University Press, Princeton, NJ, USA, 400 p

Warnock AJ, Fitter AH, Usher MB (1982) The influence of a springtail folsomia-candida (Insecta, Collembola) on the mycorrhizal association of leek allium-porrum and the vesicular-arbuscular mycorrhizal endophyte glomus-fasciculatus. New Phytol 90:285–292 doi:10.1111/j.1469-8137.1982.tb03260.x

Wurst S, Dugassa-Gobena D, Langel R, Bonkowski M, Scheu S (2004) Combined effects of earthworms and vesicular-arbuscular mycorrhizas on plant and aphid performance. New Phytol 163:169–176 doi:10.1111/j.1469-8137.2004.01106.x