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Impact of arbuscular mycorrhizal fungi and earthworms on soil aggregate stability, glomalin, and performance of pigeonpea, *Cajanus cajan*

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Abstract. Earthworms and arbuscular mycorrhizal fungi (AMF) modify soil physical and chemical properties. However, little is known about how their interactions affect water-stable aggregation, glomalin and crop performance. A greenhouse experiment was run for 9 months to test the effects of earthworms (endogeic, *Pontoscolex corethrurus*; and epigeic, *Dichogaster bolaui*) and AMF (none, *Glomus etunicatum* and *Scutellospora verrucosa*) on water-stable aggregation, glomalin levels in aggregate size classes and crop performance. The test crop was pigeonpea (*Cajanus cajan* (L.) Millsp.). The soil material used for the experiment was a humic nitisol from central Kenya mixed with sand (ratio 1 : 1). Grass residue (equivalent to 20 t ha^{-1}) was placed on top. The AMF root colonisation and external hyphal length, water-stable macroaggregates and microaggregates, total and easily-extractable glomalin in aggregate size classes, plant biomass and plant N and P uptake were measured. Earthworms were a major source of variation for soil aggregation, glomalin content and crop performance. The epigeic earthworms (*D. bolaui*) increased the amount of water-stable macroaggregates (by 10%) and glomalin in microaggregates and improved crop (growth and biomass) performance. The endogeic earthworms (*P. corethrurus*) reduced external hyphal length, root colonisation and crop performance but had no effect on water-stable aggregates and glomalin levels in in aggregate size classes. A significant AMF × earthworm interaction was observed for plant biomass and concentrations of nitrogen (N) and phosphorus (P). The AMF species together with epigeic earthworms increased plant biomass and N and P concentrations. Our results contribute to the understanding of interactions between AMF and earthworms in relation to soil aggregation, plant productivity and nutrient uptake.

Additional keywords: epigeic, endogeic, soil biota, soil fertility, soil structure, integrated soil fertility management (ISFM).

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Introduction

Integrated Soil Fertility Management (ISFM), which entails the combined use of organic amendments and mineral fertilisers to maintain soil fertility and improve nutrient use efficiency, has been proposed as a means to restore soil fertility in the (sub) tropics (Vanlauwe *et al*. 2010). The success of ISFM in terms of enhanced soil fertility depends on its effectiveness in improving chemical, physical and biological soil quality and nutrient retention. Soil biota contributes to the maintenance and productivity of agro-ecosystems by regulating nutrient cycling and improving soil structure (Kuyper and Giller 2011). In particular, earthworms and arbuscular mycorrhizal fungi (AMF) have a major influence on soil physical properties and nutrient availability (Milleret *et al*. 2009*a*).

The AMF form mutualistic symbioses with the majority of plant species, including most crops (Cardoso and Kuyper 2006). The AMF enhance uptake of phosphorus (P), zinc, copper and potassium (K) by extending the extraradical hyphae from the root surface to the soil beyond the depletion zone (Smith and Read 2008). The AMF also influence soil structure by binding and enmeshing soil particles into macroaggregates (>250 μm) and by producing glomalin (Rillig and Mummey 2006; Treseder and Turner 2007). Differential functioning of AMF depends on the species. Members of the Gigasporaceae are slower root colonisers but better soil colonisers, producing denser extraradical mycelium than members of the Glomeraceae (Hart and Reader 2002*a*). The latter factor could imply that Gigasporaceae are more important in soil structure formation

and maintenance than Glomeraceae. However, Gigasporaceae seem to be less efficient in transferring P to the host plant compared with Glomeraceae (Hart and Reader 2002*b*).

Earthworms can stimulate nutrient mineralisation and crop growth and play a major role in the build-up and maintenance of soil structure through burrowing and cast formation (van Groenigen *et al*. 2014). Earthworms ingest organic matter together with mineral soil particles, passing this mixture through their gut and excreting organo-mineral excrements (casts) that contain macroaggregates and microaggregates (Brown *et al*. 2000; Six *et al*. 2004; Pulleman *et al*. 2005). The contribution of earthworms to soil structure varies with their ecological strategy. Endogeic earthworms live in the upper layer of the mineral soil and feed on soil enriched with organic matter. They make horizontal burrows and are considered major agents of aggregation and soil organic matter stabilisation, compared with epigeic earthworms that live in the organic layer at the soil surface and rarely make burrows (Lavelle and Spain 2001).

Earthworms may influence the activity of AMF by selective feeding on spores and hyphae, damaging the hyphal network and reducing mycorrhizal effectiveness (Pattinson *et al*. 1997). Earthworms can also foster AMF dispersal through ingesting spores and hyphal fragments without digesting them, and concentrating them in faecal material (Reddell and Spain 1991; Gange 1993; Harinikumar and Bagyaraj 1994; Lee *et al*. 1996). A possible consequence of earthworms grazing on AMF is that macroaggregate and microaggregate stability in casts is (co)determined by the presence of AMF hyphae and glomalin. The AMF–earthworm interactions may also influence nutrient uptake and plant performance. However, results so far are variable, ranging from increased nutrient uptake and plant productivity (Li *et al*. 2012*a*, 2012*b*; Ma *et al*. 2006; Yu *et al*. 2005) to no effect (Eisenhauer *et al*. 2009; Milleret *et al*. 2009*a*; Xiang and Li 2014). Therefore the effects of interactions between AMF and earthworms in modifying soil structure and crop nutrition are likely influenced by AMF × earthworm species combinations, as well as the dominant nutrient limitations in the soil–plant system under study. Very few studies have investigated interactive effects of AMF and earthworms on soil aggregation (Milleret *et al*. 2009*a*, 2009*b*; Kohler-Milleret *et al*. 2013). Information on the combined impact of AMF and earthworms on glomalin pools is equally scanty.

The aim of this study was to examine the single and interactive effects of two earthworms (*Pontoscolex corethrurus* – endogeic; and *Dichogaster bolaui* – epigeic); and two AMF (*Glomus etunicatum*, now *Claroideoglomus etunicatum*) – Glomeraceae and *Scutellospora verrucosa* (now *Racocetra verrucosa* – Gigasporaceae) species on water-stable aggregates (WSA), glomalin within the aggregates and plant performance. We used pigeonpea (*Cajanus cajan* (L.) Millsp.) as the test crop. We hypothesised a positive interaction between earthworms and AMF on soil aggregation, with endogeic (*P. corethrurus*) and Gigasporaceae (*S. verrucosa*) species contributing more to soil aggregation. We further hypothesised that epigeic (*D. bolaui*) earthworms and Glomeraceae (*G. etunicatum*) species will have no effect in soil aggregation. In this regard, endogeic earthworms were expected to have a more positive effect on plant growth than epigeic earthworms, and *G. etunicatum* was expected to be more efficient in transferring P to plants than *S. verrucosa*.

Materials and methods

Soil and earthworms collection

Soil was collected at the National Agricultural Research Laboratories of the Kenya Agricultural Research and Livestock Organization Institute, Kabete (1°15′S; 36°41′E), 7 km northwest of Nairobi. The soil, a humic nitisol (FAO 1991), was collected from the upper 30 cm layer. The soil was mixed with sand collected along a river bank (ratio 1 : 1) to improve water drainage and passed through a 0.5-cm sieve to remove large particles. The main characteristics of this soil after mixing with sand were: organic carbon (C), 14.4 g kg^{-1}; total nitrogen (N), 1.1 g kg^{-1}; P (P-Olsen), 36 mg kg^{-1}; pH, 5.1; K, 1.96 cmol kg^{-1}; calcium, 11.8 cmol kg^{-1}; magnesium, 2.1 cmol kg^{-1}; sand, 41%; clay, 27%; and silt, 32%. The AMF inoculum (*Glomus etunicatum* and *Scutellospora verrucosa*, hereafter *Glomus* and *Scutellospora*) was obtained from the Kenya Forestry Research Institute at Muguga. Earthworms of species *Pontoscolex corethrurus* (hereafter *Pontoscolex*) were collected using hand-sorting from a maize field near Kenya Agricultural and Livestock Research Organization (KALRO)-Embu (0°30′N, 37°27′E) and *Dichogaster bolaui* (hereafter *Dichogaster*) was collected from a maize field near KARI-Kabete (1°15′S, 36°41′E). *Pontoscolex* (endogeic) is an introduced species in this region, and *Dichogaster* (epigeic) is native to eastern Africa (Ayuke *et al*. 2011). Earthworm species were confirmed at the National Museums of Kenya. Earthworms of similar size (young adults) were placed in containers filled with moistened soil and stored at room temperature (overnight) before inoculation.

Greenhouse experiment

A two-way factorial experiment was conducted at the National Museums of Kenya greenhouse facilities in Nairobi from December 2009 to September 2010. The temperature in the greenhouse ranged within 25–30°C. The experiment contained two factors – (1) earthworm (none, *Pontoscolex* and *Dichogaster*) and (2) AMF (none, *Glomus* and *Scutellospora*) – all tested with pigeonpea in a complete randomised design with four replicates. Treatments were labelled EN (endogeic, *Pontoscolex*), EP (epigeic, *Dichogaster*), GE (*Glomus*) and SV (*Scutellospora*), and combinations thereof. The soil–sand mixture was sterilised in an autoclave for 1 h at 121°C. Seventy-two pots (30 cm diameter, 45 cm depth) were filled with 12 kg of sterilised air dry soil each. In the treatments with AMF inoculation, 50 g of AMF inoculum was added to the surface of each pot. Pots that were not inoculated received a similar amount of steam-sterilised inoculum. In addition, all pots received 40 mL of a microbial wash to ensure similar microbiota. This was prepared from 500 g of fresh soil from the field site. The soil was suspended in 1.5 L of de-ionised water and filtered through a 25-μm mesh to eliminate AMF spores (Schroeder and Janos 2004). After adding the wash, the soil was allowed to stand for 14 days. Each of the earthworm treatments received 24 young adult earthworms (equivalent to 440 worms m^{-2}). To prevent

earthworms escaping, pots were covered by a cloth net, which was removed after 2 weeks to prevent interference with plant growth. Mulch of Rhodes grass (*Chloris gayana*) (150 g per pot, equivalent of 20 t ha^{-1}, C : N ratio of 24 : 1) was added to all pots to protect earthworms from heat and to provide a source of food. The Rhodes grass was sun-dried and chopped into 5-cm pieces. Pigeonpea was grown in two consecutive periods (first cycle, December 2009 to February 2010; and second cycle, March–August 2010) by sowing two pre-germinated seeds of pigeonpea in each period. Pigeonpea was watered as required (300 mL per day). The experiment lasted 38 weeks (9 months).

Data collection

At the end of each cycle shoots were removed, oven-dried (at 70°C) and weighed. Roots were only removed at the end of the experiment. Soil adhering to roots was carefully washed using tap water, and root fresh weight was determined. From each pot, a subsample of ~2 g of fresh roots was cut into 1-cm segments for subsequent AMF assessment (Mason and Ingleby 1998). The remaining part of roots was oven-dried at 70°C and weighed. The ratio of fresh to dry weight was determined and total root dry weight calculated. The oven-dried shoots were ground, wet-digested using Kjeldahl procedure and N and P contents in digested samples determined colourimetrically using a spectrophotometer (Anderson and Ingram 1993). Numbers of earthworms that survived were counted at the end of the experiment. On average 17 ± 4 individuals of *Pontoscolex* and 15 ± 3 individuals of *Dichogaster* were collected from pots out of the 24 earthworms initially added. We also collected juveniles and eggs of *Pontoscolex* indicating that the experimental conditions were conducive for normal earthworm activity.

Root staining, assessment of AMF colonisation and extraction of AMF hyphae

Root colonisation was assessed at the end of the experiment. A subsample of roots was stained using the modified procedure of Mason and Ingleby (1998). Briefly, roots were cleared in 2.5% KOH for 15 min at 121°C and later bleached in a mixture of 30% H_2O_2 and 30% ammonium solution (1 : 1, v : v) for 30 min to remove phenolic compounds. The roots were then acidified for 2 h with 1% HCl and stained with 0.05% acidified trypan blue dissolved in glycerol–water (1 : 1, v : v) for 3 min at 121°C. Estimation of AMF colonisation was according to Trouvelot *et al*. (1986). Thirty root fragments were mounted on two slides each containing 15 root fragments. The fragments were observed under the microscope (magnification 160–400×) for the presence of hyphae, arbuscules and vesicles. Since the percentages for AMF colonisation by hyphae, arbuscules and vesicles were highly correlated, we present only data for total AMF colonisation.

The AMF hyphal length was also assessed at the end of the experiment. Hyphae were extracted from a 10-g soil subsample by an aqueous extraction and membrane filter technique following Jakobsen *et al*. (1992). Soil samples were mixed and suspended in 100 mL of deionised water, to which 12 mL of a sodium hexametaphosphate solution was added. The soil suspension was shaken for 30 s (end-over-end), left on the bench for around 30 min and then decanted through a 45-µm sieve to retain hyphae, roots and organic matter. Material on the sieve was sprayed gently with deionised water to remove clay particles, and then transferred into a 250-mL flask with 200 mL of deionised water. The flask was shaken vigorously by hand for 5 s, left on the bench for 1 min, and then a 2-µL aliquot was taken and pipetted onto a 25 mm diameter Millipore filter (25-µm pore size). The material on the filter was stained with 0.05% Trypan Blue and transferred to microscope slides. Hyphal length was measured with the grid-line intersect method at 100× magnification. Observations confirmed that non-mycorrhizal treatments remained free of AMF.

Assessment of water-stable macroaggregates and microaggregates

The separation of aggregates into separate size classes of WSA was carried out using the manual wet-sieving method described by Elliott (1986). Briefly, a subsample of 80 g was spread evenly onto a 2000-µm sieve, immersed in distilled water and left to slake for 5 min before starting the sieving process. Then, aggregates were separated by moving the 2000-µm sieve up and down by ~3 cm with 50 repetitions in 2 min. The aggregates >2000 µm were collected as large macroaggregates and the same sieving procedure was repeated for the 2000–250 µm fraction with the 250-µm sieve to give small macroaggregates. Then, the fraction 53–250 µm was obtained by sieving with 53-µm sieve as free microaggregates (Mi). The aggregates remaining on top of each sieve were backwashed into labelled and preweighed containers and dried at 60°C overnight before final weight was recorded. Soil material that passed through 53 µm was determined by taking a 300-mL subsample from the supernatant water of the whole volume after thoroughly shaking the suspension, and dried in the same way as the other fractions. The weights were then corrected for the size of the subsample as compared with the whole volume and the fractions were recorded as free silt and clay (SC). All soil fractions except silt and clay were corrected for sand content. A subsample of each fraction was dispersed using sodium hexametaphosphate, and sand was isolated after wet-sieving as above (Márquez *et al*. 2004). The proportion of sand in various aggregates was 0.03 in large macroaggregates, 0.40 in small macroaggregates and 0.07 in microaggregates. The large and small macroaggregate fractions were combined as total macroaggregates (TMa).

Assessment of glomalin

Glomalin extraction from TMa and Mi was carried out as described by Wright and Upadhyaya (1998). Easily-extractable glomalin (EEG) was extracted with 20 mM citrate, pH 7.0 at 121°C for 30 min. Total glomalin (TG) was extracted with 50 mM citrate, pH 8.0 at 121°C in rounds of 60 min each. For the sequential extractions, the supernatant was removed by centrifugation at 5000*g* for 20 min. Extraction of a sample was done till the supernatant showed none of the red-brown colour typical of glomalin, and glomalin was determined by the Bradford assay (Wright and Upadhyaya 1996, 1998). To account for differences in amount of sand (with which there is no glomalin associated), the glomalin content of

each fraction (*f*) was expressed on a sand-free basis, calculated as follows:

$$Glomalin\left(\frac{\text{mg}}{\text{g}}\text{sand}-\text{free fraction}\right)=\frac{Glomalin(f)\left(\frac{mg}{g}\right)}{1-(Sand(f)proportion)}$$

Data analysis

Effects of AMF (factor with three levels: control, *Glomus* and *Scutellospora*) and earthworms (factor with three levels: control, endogeic- *Pontoscolex* and epigeic-*Dichogaster*) on shoot and root biomass, N and P uptake, N : P ratio, aggregate size distribution and glomalin in aggregate fractions were analysed using two-way ANOVA. The AMF hyphal length and root colonisation were analysed only for treatments with AMF. Post-hoc analysis was performed whenever a significant effect ($P < 0.05$) was observed using Fisher's Least Significant Difference. The relationship between mycorrhizal parameters, soil aggregation and glomalin contents were tested by Pearson correlation. All statistical analyses were performed using GENSTAT 14th Edition software (VSN international) except for Pearson correlation, which was carried out with SPSS (PASW Statistics 19).

Results

Extraradical hyphal length and mycorrhizal root colonisation

There were no mycorrhizal structures in roots of pigeonpea and no aseptate hyphae were observed in pots without mycorrhizal inoculum. Mycorrhiza extraradical hyphal length (MEH) and percentage root length colonised by AMF (%RLC) were significantly affected by earthworm and AMF × earthworm interactions ($P < 0.05$). The AMF had no effect on either MEH or RLC ($P > 0.05$, Fig. 1). Presence of endogeic earthworms (*Pontoscolex*) reduced MEH and RLC by more than 25% compared with treatments with no earthworms (control) (Fig. 1). Epigeic earthworms (*Dichogaster*) had no effect on MEH and RLC ($P > 0.05$, Fig. 1).

WSA

The recovery rate of WSA ranged within 98–100%, indicating minimal losses of aggregates during wet-sieving. The WSA were corrected for sand (Márquez *et al*. 2004). Sand-free water-stable macroaggregates and microaggregates are presented. Water-stable macroaggregates and microaggregates and silt and clay were significantly affected by earthworm and

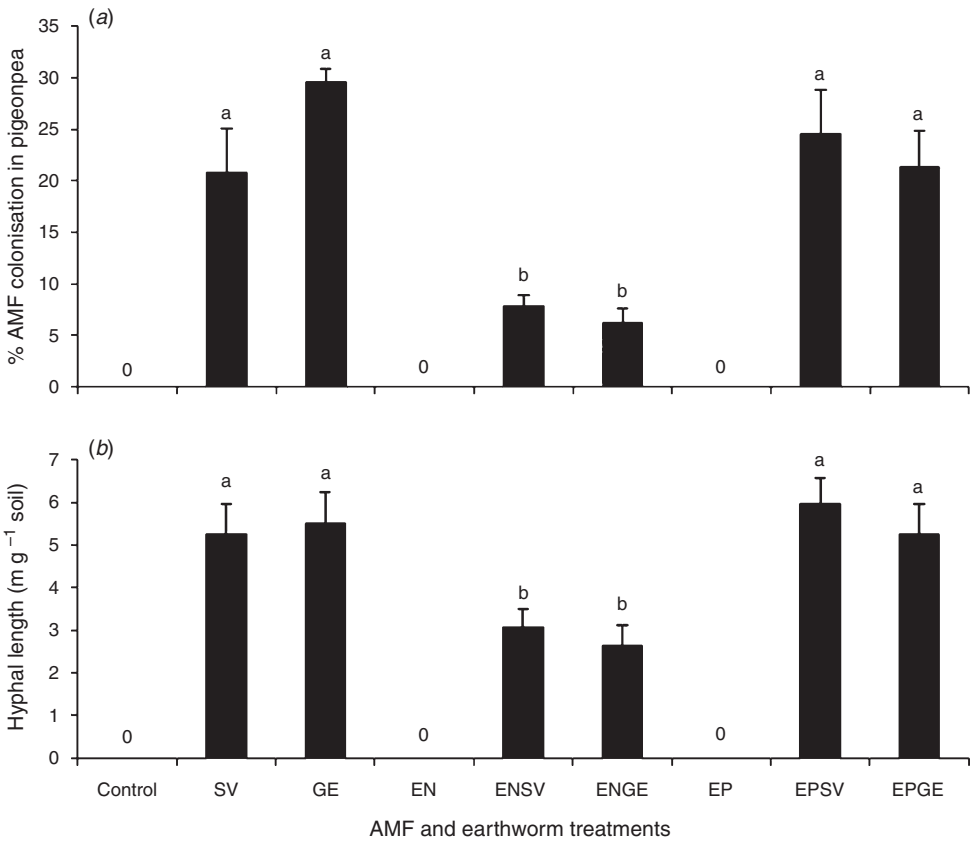

Fig. 1. Effect of arbuscular mycorrhizal fungi (AMF) and earthworm species on fractional root colonisation (*a*) and extraradical hyphal length (*b*). Hyphal length was affected by earthworms ($F = 21.60$, $P < 0.001$) and earthworm–AMF interaction ($F = 4.89$, $P = 0.05$); and root colonisation was affected by earthworms ($F = 11.73$, $P = 0.001$) and earthworm–AMF interaction ($F = 9.64$, $P = 0.001$). Errors bars are standard error (SE). Means followed by the same letters are not significantly different at $P < 0.05$. Treatments are labelled EN (endogeic, *Pontoscolex*), EP (epigeic, *Dichogaster*), GE (*Glomus*) and SV (*Scutellospora*), and combinations thereof.

AMF × earthworm interactions ($P < 0.05$, Table 1). The AMF was not a significant source of variation for all WSA ($P > 0.05$, Table 1). Generally, epigeic earthworms increased water-stable macroaggregates (49%) and reduced microaggregates (16%) compared with treatments with no earthworms (46% macroaggregates and 19% microaggregates) and those with endogeic earthworms (45% macroaggregates and 20% microaggregates). Similarly epigeic earthworms in presence of AMF (EPSV and EPGE) increased levels of macroaggregates by ~8% and reduced levels of microaggregates and silt and clay by 24% and 40% respectively compared with treatments without earthworms (Fig. 2). Alternatively, endogeic earthworms in presence of AMF (ENSV and ENGE) had no significant effect on all WSA compared with control treatments ($P > 0.05$ in all cases), but showed significantly lower levels of macroaggregates compared with treatments with AMF and endogeic earthworms alone (Fig. 2). Epigeic earthworms alone had no effect on levels of WSA (Fig. 2).

Glomalin in aggregates

The TG and EEG were generally higher in microaggregates than in macroaggregates. The TG and EEG in various aggregates were significantly affected by earthworms and earthworm × AMF interactions ($P < 0.05$, Table 1). The AMF had no significant effect on TG and EEG in aggregates ($P > 0.05$ in all cases, Table 1).

The TG in macroaggregates was significantly higher in treatments with epigeic earthworms in combination with AMF (EPSV and EPGE) compared with control ($P < 0.05$). Presence of endogeic earthworms alone (EN) or combined with AMF (ENSV and ENGE) had no significant effect ($P > 0.05$, Fig. 3). In microaggregates, TG was significantly higher in treatments with epigeic earthworms alone (EP) as well as both earthworm treatments in combination with *Scutellospora* (ENSV and EPSV, Fig. 3). All other treatments had no effect on levels of TG in aggregates ($P > 0.05$ in all cases).

The EEG in macroaggregates was significantly higher in treatments with *Scutellospora* alone (SV), and significantly lower in treatments with endogeic earthworms alone (EN) and treatments with endogeic earthworms combined with *Scutellospora* (ENSV, $P < 0.05$, Fig. 3). Epigeic earthworms alone or in presence of AMF had no effect on EEG in macroaggregates compared with control ($P > 0.05$, Fig. 3). However, treatments with epigeic earthworms alone (EP) and treatments with epigeic earthworms combined with AMF (EPSV and EPGE) showed higher levels of EEG in macroaggregates than in treatments with endogeic earthworms alone or endogeic with AMF (ENSV and ENGE, Fig. 3). In microaggregates, the EEG trend was different. All treatments had increased levels of EEG in microaggregates above controls ($P < 0.005$, Fig. 3). The EEG in microaggregates was highest in treatments with endogeic earthworms in combination with *Glomus* (ENGE) followed by treatments with epigeic earthworms alone (EP, Fig. 3).

Pigeonpea growth and biomass

The experiment ran for 9 months to maximise the chance of recording significant changes in WSA fractions and glomalin content in these aggregates. The duration of the experiment necessitated two harvests for pigeonpea. The aboveground materials were removed during the first harvest but the roots remained in the mesocosms. Here we present data for the two harvesting periods: the first in February and the second in August. Pigeonpea growth and biomass were only affected by earthworms ($P < 0.05$) in the first harvest but AMF and AMF × earthworm interaction had no effect (Table 2). However, during the second harvest, pigeonpea growth and biomass was significantly affected by earthworms and AMF × earthworm interaction ($P < 0.05$, Table 2). The AMF had no effect in the two harvest periods (Table 2). During the first harvest, treatment with epigeic earthworms had the highest growth (115 cm) and biomass (45 g), followed by treatments with no earthworms (75 cm and 29 g) and the least growth and biomass were in treatments with endogeic earthworms (55 cm and 12.3 g). During the second harvest, treatments with epigeic earthworms alone (EP) or in combination with AMF (EPSV and EPGE) had the highest level of growth and biomass followed by treatments with AMF alone (Table 2). Treatments with endogeic earthworms alone (EN) or in combination with AMF (ENSV and ENGE) had the lowest growth (Table 2).

Nutrient concentration in pigeonpea

Nutrient concentration (N and P) and N : P ratio were significantly affected by earthworms and AMF × earthworm interaction during the first harvest ($P < 0.05$, Table 2). However, during the second harvest only AMF × earthworm interaction was a significant source of variation for P concentration. The AMF had no effect in the two harvest periods (Table 2).

Treatments with epigeic earthworms alone (EP) or in combination with AMF (EPSV and EPGE) as well as treatments with endogeic earthworms in combination with *Glomus* (ENGE) increased the N concentration in pigeonpea compared with control during the first harvest (Table 2).

Table 1. Results of the two-way ANOVA of the effects of AMF and earthworms on sand-free water-stable aggregate fractions and total (TG) and easily-extractable (EEG) glomalin in macroaggregates (TM), microaggregates (Mi) and silt and clay (SC)

F-values are followed by *P*-values (between parentheses). Values in bold have $P < 0.05$

	Water-stable aggregate (%)			Glomalin in WSA (mg g^{-1})			
	TM	Mi	SC	TG		EEG	
Treatments	>250 µm	53–250 µm	<53 µm	>250 µm	53–250 µm	>250 µm	53–250 µm
AMF	0.60(0.52)	0.68(0.55)	1.53(0.23)	**3.60(0.04)**	0.52(0.60)	2.25(0.13)	**6.92(0.00)**
Earthworm	**7.43(0.00)**	**4.64(0.02)**	**13.81(0.00)**	**14.86(0.00)**	**9.55(0.00)**	**21.41(0.00)**	**15.48(0.00)**
AMF × earthworm	**3.60(0.018)**	**2.67(0.05)**	**3.15(0.03)**	**4.16(0.01)**	**2.73(0.05)**	**9.26(0.00)**	**8.19(0.00)**

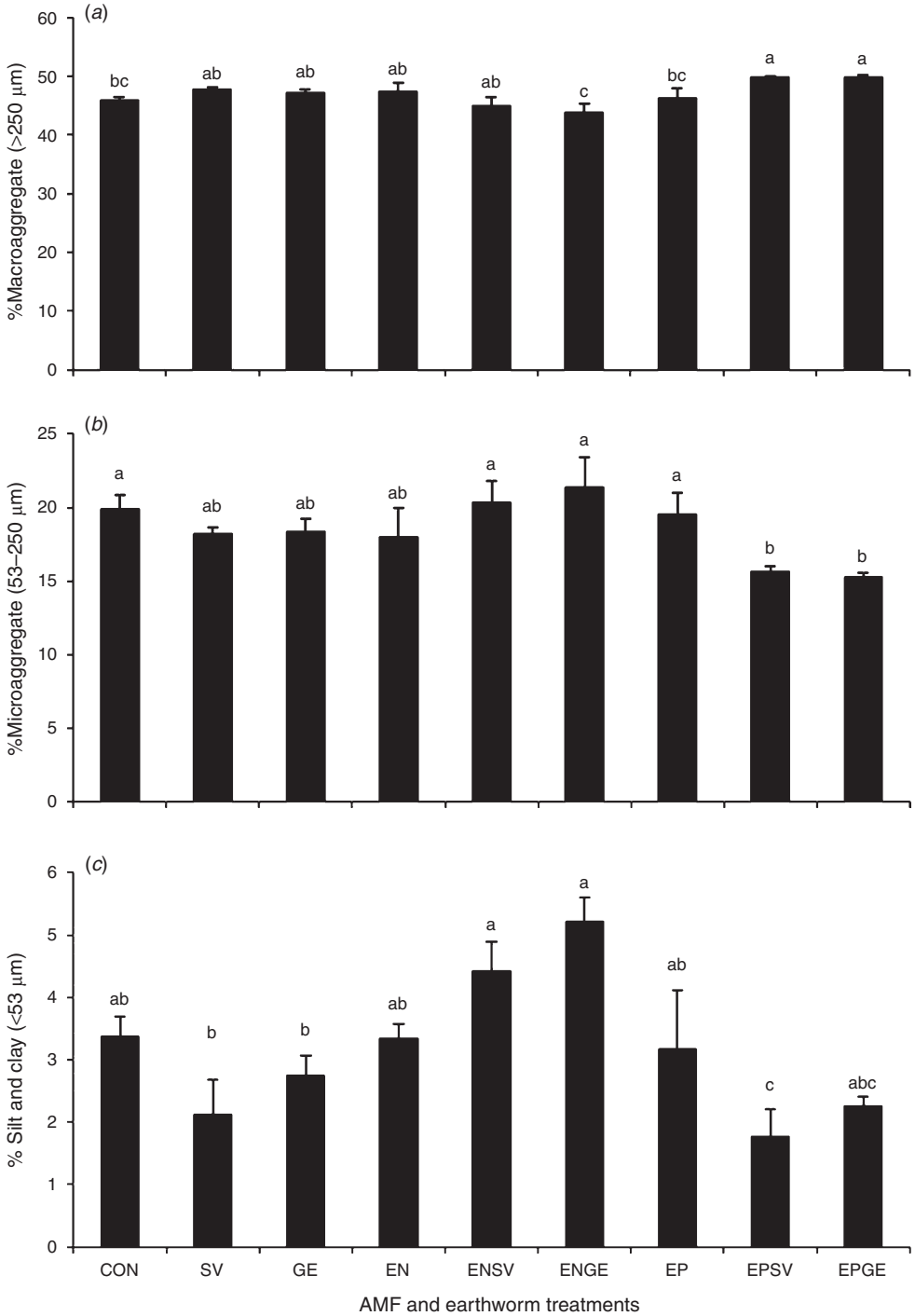

Fig. 2. Effect of arbuscular mycorrhizal fungi (AMF) and earthworm species on sand-free water-stable (*a*) macroaggregates (>250 µm), (*b*) microaggregates (250–53 µm) and (*c*) silt and clay (<53 µm). Errors bars are standard error (SE). Means followed by the same letters are not significantly different at $P < 0.05$ Treatments are labelled EN (endogeic, *Pontoscolex*), EP (epigeic, *Dichogaster*), GE (*Glomus*) and SV (*Scutellospora*), and combinations thereof.

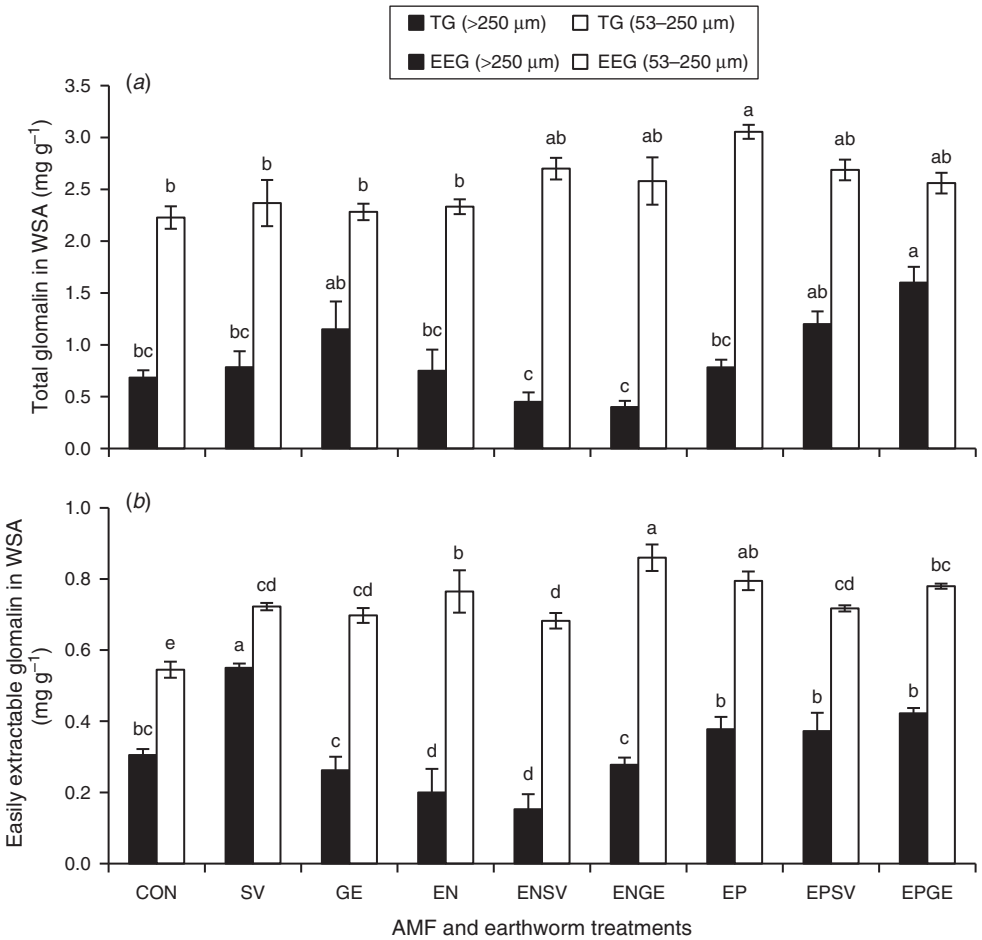

Fig. 3. Effect of arbuscular mycorrhizal fungi (AMF) and earthworms on (*a*) total and (*b*) easily-extractable glomalin (TG and EEG) in macroaggregates (>250 μm) and microaggregates (250–53 μm). Errors bars are standard error (SE). Mean followed by same letters in the same parameters are not significantly different at $P < 0.05$. Treatments are labelled EN (endogeic, *Pontoscolex*), EP (epigeic, *Dichogaster*), GE (*Glomus*) and SV (*Scutellospora*), and combinations thereof. WSA is water-stable aggregates.

Endogeic earthworms alone (EN) or combined with *Scutellospora* (ENSV) had no effect on N concentration. The AMF alone (SV and GE) reduced N concentrations in pigeonpea, with a significant effect observed in treatments with *Glomus* (GE, Table 2).

Endogeic earthworms alone (EN) increased P concentration in pigeonpea, and epigeic earthworms alone (EP) or combined with AMF (EPSV and EPGE) reduced P concentration compared with control during the first harvest (Table 2). Treatments with AMF (SV and GE) alone had no effect on P concentration compared with control, but showed higher P concentration than treatments with epigeic earthworms alone or in combination with AMF during first harvest (Table 2). During the second harvest, treatments with *Glomus* (GE) alone and those with epigeic earthworms in combination with *Scutellospora* (EPSV) increased P concentration but treatments with endogeic earthworms in combination with *Scutellospora* (ENSV) reduced P concentrations in pigeonpea (Table 2).

The N : P ratio was almost always below 10 in all treatments. The N : P ratio was not affected by AMF, but was affected by earthworms and AMF × earthworm interactions only in the first cycle (Table 2). Earthworms species alone or combined with AMF increased N : P ratio in the first but not the second cycle.

Correlations between parameters

The correlation matrix between several measured variables is shown in Table 3. The AMF hyphal length was significantly positively correlated with %RLC ($r = 0.95$, $P < 0.01$). The AMF hyphal length was also significantly correlated with water-stable macroaggregates ($r = 0.90$), water-stable microaggregates ($r = –0.86$), silt and clay ($r = –0.97$) and variables of plant performance. The AMF hyphal length was also significantly positively correlated with TG in macroaggregates. The TG in macroaggregates was positively correlated with water-stable macroaggregates, and negatively correlated with water-stable microaggregates. The EEG showed no significant correlations with WSA of various size classes (Table 3).

Table 2. Effects of AMF and earthworms on height, shoot and root dry biomass, N and P concentrations and N : P ratio in pigeonpea

Different letters in each column indicate significant differences between treatments at $P < 0.05$. Standard errors are provided between the parentheses. Means followed by the same letters in the same parameters are not significantly different at $P < 0.05$. The lower part of the table shows F-values, followed by *P*-values (between parentheses). Values in bold have $P < 0.05$. Treatments are labelled EN (endogeic, *Pontoscolex*), EP (epigeic, *Dichogaster*), GE (*Glomus*) and SV (*Scutellospora*), and combinations thereof

	Height		Dry weight			Pigeonpea (g kg^{-1} plant biomass)					
			Shoot		Root	N		P		N : P	
Treatments	Feb	Aug	Feb	Aug	Aug	Feb	Aug	Feb	Aug	Feb	Aug
No earthworm	55.75(6.9)c	94.38(9.1)c	3.8(0.3)a	7.9(1.0)d	6.1(1.2)cd	22.9(0.7)b	21.0(1.4)a	3.4(0.1)b	3.1(0.5)b	6.8(0.3)b	7.4(1.4)a
SV	84.38(3.9)b	137.56(9.8)bc	11.0(1.1)a	27.5(2.9)b	7.1(1.3)c	19.3(2.3)b	20.1(2.2)a	3.5(0.3)b	3.0(0.4)b	5.7(1.0)b	7.4(1.5)a
GE	86.00(10.5)b	148.09(5.8)bc	8.3(1.7)a	27.6(2.6)b	6.0(0.9)cd	16.0(0.9)c	23.6(2.6)a	3.4(0.4)b	3.9(0.6)a	5.0(0.7)b	6.5(1.2)a
Endogeic earthworm	55.63(8.9)c	121.75(8.5)c	2.0(0.1)a	16.2(2.0)c	3.1(0.2)e	21.0(1.4)b	21.0(5.2)a	4.1(0.3)a	3.6(0.3)ab	5.2(0.5)b	6.0(1.4)a
ENSV	62.13(3.9)a	57.00(3.42)d	3.3(0.9)a	5.6(0.3)d	4.9(1.00)de	22.8(1.0)b	16.6(1.7)a	2.2(0.1)d	2.5(0.2)c	9.3(1.1)a	6.7(0.6)a
ENGE	47.25(9.9)c	59.63(10.9)d	3.3(0.5)a	6.6(1.3)d	6.1(1.1)cd	25.0(1.5)a	21.4(1.5)a	2.5(0.3)c	3.0(0.2)b	11.3(0.6)a	7.4(0.7)a
Epigeic earthworm	119.38(6.9)a	144.56(8.5)bc	6.0(1.2)a	39.9(7.2)a	16.1(0.9)a	24.1(1.5)a	17.5(2.9)a	2.4(0.2)c	2.7(0.3)bc	10.3(1.2)a	6.5(0.4)a
EPSV	103.50(12.2)ab	177.63(5.0)ab	12.0(1.4)a	38.7(3.2)a	13.2(0.3)b	24.5(1.4)a	21.9(0.9)a	2.7(0.2)c	3.8(0.3)a	9.4(0.4)a	5.8(0.4)a
EPGE	122.50(14.6)a	167.00(6.4)b	8.3(3.0)a	29.8(4.3)b	10.9(1.1)c	25.4(1.7)a	16.6(2.2)a	2.6(0.1)c	3.0(0.3)b	9.5(1.0)a	5.8(1.0)a
ANOVA											
AMF (A)	0.58(0.57)	0.18(0.84)	**9.3(0.00)**	0.6(0.57)	0.6(0.53)	0.1(0.88)	0.1(0.89)	**4.0(0.03)**	0.2(0.82)	1.5(0.23)	0.0(1.0)
Earthworm (E)	**28.26(0.00)**	**50.97(0.00)**	**15.8(0.00)**	**46.9(0.00)**	**69.4(0.00)**	**10.4(0.00)**	1.0(0.380)	**10.1(0.001)**	0.5(0.60)	**19.9(0.00)**	0.8(0.47)
A × E	1.97(0.13)	**11.70(0.00)**	1.5(0.24)	**8.1(0.00)**	**4.8(0.01)**	**3.8(0.01)**	1.3(0.29)	**7.3(0.00)**	**3.1(0.03)**	**7.5(0.00)**	0.4(0.83)

Table 3. Correlation matrix (Pearson) for correlations between various parameters

$n = 9$ for correlations between water-stable macroaggregates (>250 µm), microaggregate (53–250 µm), silt and clay (<53 µm), dry weight (shoot and root), nutrient concentration (nitrogen and phosphorus), total (TG) and easily-extractable (EEG) glomalin content in various aggregates fractions (>250 and 53–250 µm). $n = 6$ for correlations with AMF parameters (% root length colonised by AMF (%RLC) and AMF hyphal length (MEH)). Values in bold are significant; * significant at $P < 0.05$; ** significant at $P < 0.01$

		Water-stable aggregates			Height		Dry weight		Nitrogen		Phosphorus		TG		EEG		AMF
		>250 µm	53–250 µm	>53 µm	Feb	Aug	Shoot	Root	Feb	Aug	Feb	Aug	>250 µm	53–250 µm	>250 µm	53–250 µm	MEH
WSA	53–250 µm	**–0.99****															
	>53 µm	**–0.92****	**0.85****														
Height	Feb	**0.76***	**–0.72***	**–0.79***													
	Aug	**0.91****	**–0.87****	**–0.92****	**0.87****												
Dry weight	Shoot	**0.75***	**–0.70***	**–0.83****	**0.93****	**0.92****											
	Root	0.42	–0.38	–0.44	**0.83****	0.60	**0.77***										
Nitrogen	Feb	0.01	–0.05	0.22	0.10	–0.12	–0.06	0.47									
	Aug	–0.02	0.03	–0.09	–0.24	0.11	0.01	–0.31	–0.52								
Phosphorus	Feb	0.19	–0.15	–0.29	–0.27	0.19	–0.06	–0.53	–0.63	0.57							
	Aug	0.48	–0.48	–0.46	0.12	0.52	0.32	–0.12	–0.46	**0.83****	0.58						
TG	>250 µm	**0.89****	**–0.91****	**–0.79***	**0.79***	**0.87****	**0.69***	0.44	–0.01	–0.02	0.05	0.45					
	53–250 µm	–0.04	0.05	0.11	0.43	0.08	0.35	**0.75***	0.58	–0.58	**–0.78***	–0.49	–0.06				
EEG	>250 µm	0.49	–0.42	–0.64	0.63	0.59	**0.67***	0.51	0.057	–0.09	0.03	–0.06	0.42	0.07			
	53–250 µm	–0.02	–0.04	0.21	0.12	0.08	0.20	0.26	0.30	–0.15	–0.29	–0.08	0.03	0.49	0.10		
AMF	MEH	**0.90***	**–0.86***	**–0.97****	**0.92****	**0.97****	**0.98****	0.66	–0.33	0.30	0.62	0.70	**0.82***	–0.38	0.58	–0.42	
	%RLC	0.77	–0.73	**–0.87***	**0.81***	**0.91***	**0.88***	0.47	–0.53	0.45	0.71	0.80	0.79	–0.57	0.42	–0.43	**0.95****

Discussion

The purpose of this study was to elucidate the combined effect of AMF and earthworms on soil aggregation, glomalin levels in aggregates and crop performance. We hypothesised positive interaction between earthworms and AMF in on soil aggregation, with endogeic (*Pontoscolex*) and Gigasporaceae (*Scutellospora*) species contributing more to soil aggregation compared with epigeic (*Dichogaster*) and Glomeraceae (*Glomus*) species.

Crop performance and N and P concentrations

There was an N-limitation in this study. Although pigeonpea can increase N availability through biological N_2 fixation and N mineralisation of dead roots (C : N ratio = 12, Sakala *et al*. 2000), the N : P ratio (Table 2) indicated N-limitation. Plant N : P ratios have been used to assess whether N or P is limiting growth. Güsewell (2004) concluded that N : P < 10 indicates N-limitation. Johnson *et al*. (2010) and Li *et al*. (2012*a*) have shown impaired AMF functioning in N-limited soil due to competition for N between plant and AMF. Hodge and Fitter (2010) reported competition for N between AMF and plants. The soil in our experiment was clayey and most of the C and N was likely physically and chemically protected (Vanlauwe *et al*. 2006). The N availability due to mineralisation of soil organic matter is therefore likely to be very low. At the same time P availability in this soil was adequate. High available P levels (P-Olsen) have been reported in nitisols in Kenya with a history of P fertilisation (Murage *et al*. 2000; Kimetu *et al*. 2006). The resulting strong N-limitation with P-sufficiency can create conditions under which the beneficial effect of mycorrhiza on plant performance is limited. Lack of significant correlations between AMF parameters (root colonisation and extraradical hyphal length) and N uptake may indicate that AMF did not contribute to N acquisition. Severe N-limitation has also been observed in field experiments in Kabete (Janssen 2011). Because the mycorrhizal role in nutrient uptake is more important for poorly mobile nutrients such as P (where diffusion is the main mechanism for uptake) than for nutrients such as N (where mass flow is the main mechanism) it is not surprising that the mycorrhizal benefits were low and declined with subsequent harvests.

This study showed positive effects of epigeic earthworms (*Dichogaster*) and AMF on plant performance. The results are in agreement with previous results showing positive effects of epigeic earthworms (*Eisenia fetida*) on maize biomass (Li *et al*. 2012*b*). *Dichogaster* also positively influenced N availability possibly through ingestion and decomposition of organic matter (grass mulch placed on the surface). *Dichogaster* also had no negative effect on AMF hyphal length and root colonisation (Table 1; Fig. 1), in line with its ecological behaviour as a litter forager forming little burrows (5–10 cm) within the soil (Sahu *et al*. 1988; Kale and Karmegam 2010). A positive effect of both epigeic and anecic earthworms (*Lumbricus rubellus* and *L. terrestris*) on abundance of AMF was reported by Dempsey *et al*. (2013). *Dichogaster* and AMF increased nutrient supply via different mechanisms, and additively improved nutrient uptake and plant performance. *Dichogaster* improved nutrient supply through ingestion of residue placed on the surface and incorporation of partly decomposed residues into casts (Bossuyt *et al*. 2006), while AMF enhanced crop nutrition by extending the extraradical hyphae from the root surface to the soil beyond the depletion zone (Smith and Read 2008).

Negative effects of *Pontoscolex* on AMF and performance of the mycorrhiza-responsive pigeonpea coincides with results from studies by Tuffen *et al*. (2002), Eisenhauer *et al*. (2009) and Milleret *et al*. (2009*b*). Our results, however, contrast with other studies that showed positive effects of other endogeic earthworm × AMF interactions on crop performance and nutrition (Ma *et al*. 2006; Li *et al*. 2012*a*, 2013*a*, 2013*b*). Several factors could explain these contradictory results. *Pontoscolex* negatively affected AMF functioning by reducing hyphal length through mechanical disruption of the mycorrhizal network. Negative effects of endogeic earthworms on AMF hyphal lengths are common (Pattinson *et al*. 1997; Tuffen *et al*. 2002; Ortiz-Ceballos *et al*. 2007). Tuffen *et al*. (2002) found that mechanical disruption of the extraradical network by earthworms eliminated the effect of AMF on ^{32}P transfer between mycorrhizal plants. In addition, *Pontoscolex* produced a very compact impermeable superficial layer in the absence of residue, which affects structural pore volume and infiltration (Blanchart *et al*. 2004). We observed excess water on the surface of the mesocosms with *Pontoscolex*, despite superficial residue addition. Increased water logging, poor aeration, increased bulk density and reduced AMF activity thus led to negative effects of *Pontoscolex* + AMF on plant performance.

Soil aggregation

To our surprise, changes in aggregate size distribution due to *Pontoscolex* were very limited. It is likely that *Pontoscolex* had little access to residues on the surface, and mainly fed on soil organic matter, thereby disrupting existing aggregates and probably making new aggregates. Under such conditions, *Pontoscolex* may not have contributed to the formation of stable macroaggregates. Similar results were shown by Milleret *et al*. (2009*a*), who reported no significant effect of earthworms on stable-aggregate size distribution using *Allolobophora chlorotica* and leek (*Allium porrum*). Milleret *et al*. (2009*b*) pointed out that in addition to their burrowing habit, the effect of earthworms on soil compaction (compacting versus decompacting species) is important. Compacting species (e.g. *Pontoscolex* and *Allolobophora chlorotica*) negatively affect soil aggregation by decreasing structural pore volumes and disappearance of structural pore radii (Blanchart *et al*. 2004). Taken together, our results and those of other researchers (Milleret *et al*. 2009*b*) contrast with studies that showed that endogeic earthworms increase WSA (Six *et al*. 2004). Our experiment was carried out in a greenhouse under controlled conditions where only one earthworm species was added, and where no residues were incorporated in the soil but placed on the soil surface. Giannopoulos *et al*. (2010) found a positive effect of endogeic earthworms (*Aporrectodea caliginosa*) on soil aggregation when residues were incorporated in the soil but not when placed on top. Also, under natural (field) conditions, earthworm species richness is larger than one and species mixtures of various functional groups (including compacting and decompacting species) co-occur.

Positive effects of epigeic earthworms (*Dichogaster*) alone or in combination with AMF on formation of water-stable macroaggregates were observed. Although these changes were relatively small (Fig. 2), the results may indicate potential for stronger effects under circumstances in which aggregate stability is low (disturbed soil). This effect could be attributed to ingestion and incorporation of partly decomposed residues into their casts. Earthworms incorporated grass mulch placed on the soil surface in the aggregates, resulting in enhanced aggregate stability. A decline in the fraction of microaggregates and silt and clay (Fig. 2) indicates that some of the original microaggregates were bound to form macroaggregates. Taken in this perspective, our results are in agreement with those of Bossuyt *et al*. (2006) and Giannopoulos *et al*. (2011), showing improved soil aggregation and incorporation of residue-derived organic matter in the aggregates when epigeic earthworms were added and residue placed on the surface. Our differences were, however, smaller than in the study by Bossuyt *et al*. (2006), who observed a larger proportion of fresh residue in both macroaggregates and microaggregates within macroaggregates. This could be because we did not crush the original macroaggregates, in contrast to Bossuyt *et al*. (2006).

Presence of AMF did not result in consistent differences among the various aggregate fractions. However, positive correlations between macroaggregates and extraradical hyphal length as well as macroaggregates with TG in macroaggregates (Table 3) support the positive contribution by AMF on soil aggregation. The effect of AMF on soil aggregation is often masked by indirect effects of plants through roots (Rillig *et al*. 2002; Piotrowski *et al*. 2004; Hallett *et al*. 2009; Milleret *et al*. 2009*b*; Kohler-Milleret *et al*. 2013). We suggest that indirect effects of pigeonpea roots on soil aggregation masked the effect of AMF. Further studies are desirable to understand AMF × plant interaction on soil aggregation. *Scutellospora* showed higher hyphal length than *Glomus*, confirming earlier reports that *Scutellospora* is a better soil coloniser than *Glomus* (Hart and Reader 2002*a*). However, it was not better at soil aggregation than *Glomus*.

Our hypothesis regarding the interactive role of AMF and earthworms in increasing WSA was rejected since this effect could not be generalised for earthworm species. We attribute the lack of such interactive effect due to negative effect of *Pontoscolex* on AMF activity, as shown by reduced AMF external mycelium and root colonisation of both plants (Fig. 1). Although *Dichogaster* had no negative effect on AMF hyphal length and fractional root colonisation, lack of a main mycorrhizal effect on soil aggregation and indirect effects of plants through roots may have influenced this observation.

Glomalin in aggregates

The nature of glomalin is still contested. Whereas some authors consider glomalin (as assessed through the Bradford assay) as indicative for AMF (Treseder and Turner 2007; Koide and Peoples 2013), other authors have suggested that several glycoproteins and humic materials are co-extracted and therefore prefer the acronym GRSP (glomalin-related soil protein) (Schindler *et al*. 2007; Gillespie *et al*. 2011). Nie *et al*. (2007) measured substantial increases in glomalin contents after addition of rice straw, confirming that humified plant material may end up in this operationally defined pool.

In this study, epigeic (*Dichogaster*) earthworms increased TG and EEG in both aggregate fractions (Fig. 3), suggesting that *Dichogaster* stimulated incorporation of residue-derived organic matter into the mineral soil reflected by increasing levels of occluded glomalin in stable aggregates. Under such conditions, decomposed residues in casts could have been intimately mixed with mineral soil in the process of biogenic aggregate formation contributing to the glomalin pool. Contents of EEG in microaggregates were consistently higher with *Dichogaster* than with *Pontoscolex*. Earthworms could influence glomalin pools in various ways. They can increase glomalin production and physical protection from decomposition through incorporation in aggregates. *Dichogaster* may have influenced both glomalin production and stabilisation by increasing WSA and improving AMF functioning. *Pontoscolex* may have influenced glomalin by disrupting existing aggregates.

Higher glomalin contents in microaggregates than macroaggregates coincide with findings by Wright *et al*. (2007). The soil of our experiment was collected from regularly tilled soil. Since macroaggregates change with management (Six *et al*. 2000), this suggests that microaggregates are more stable in storing glomalin than macroaggregates. Microaggregates also have higher C contents than macroaggregates (Green *et al*. 2005; Gulde *et al*. 2008), indicating their likely role in glomalin stabilisation.

Conclusion

Our study highlights the role of AMF and earthworms, and their interactions on soil aggregation. Our initial hypothesis regarding the strong positive effects of the combination of endogeic earthworms (*Pontoscolex*) and *Scutellospora* was rejected. The endogeic earthworms (*Pontoscolex*) reduced mycorrhizal colonisation and external hyphal length, resulting in lower crop performance and glomalin contents. The absence of sufficient organic material in the soil (rather than at the soil surface) resulted in soil compaction and excess water, and this may also have negatively affected crop performance. The epigeic earthworms (*Dolichogaster*) contributed more to glomalin than *Pontoscolex*. The chemical properties of the Kabete soil with relatively high P content likely created conditions where the role of earthworms in mobilising N may have been more important than the role of AMF in enhancing P uptake.

Conflicts of interest

The authors declare no conflicts of interest.

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