Roots and earthworms under grass, clover and a grass-clover mixture

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Abstract
White clover has a lower root biomass and a higher abundance of earthworms than grass. This might have an impact on the ecosystem services soil structure maintenance and water regulation when white clover is introduced in a grass-clover mixture. We investigated the root biomass, the abundance of earthworms and a selection of soil physical parameters in white clover, grass-clover, and grass with and without inorganic N fertilizer. The treatment with clover-only had a lower root biomass, a lower C/N-ratio of the roots, a higher abundance of earthworms, a higher number of earthworm burrows, a lower penetration resistance at the 20-30 cm soil layer and a lower proportion of crumbs in the soil, than the other treatments. This confirms the literature that pure clover stimulates the ecosystem services of water regulation, but is less conducive to soil structure maintenance. However, the grass-clover mixture did not differ significantly from the grass treatments, but differed from pure clover in a higher percentage of soil crumbs. We infer that, when clover is introduced in grassland to reduce the reliance on inorganic fertilizer, the mixture of grass and clover maintains the positive impact of grass roots on soil structure but only may show a positive effect of clover-only on water regulation with a higher clover percentage in the dry matter than in our experiment.

Key Words
Roots, earthworms, grassland, clover, ecosystem services.

Introduction
In sustainable grassland the focus is on ecosystem services like soil structure maintenance and water regulation, because of the perennial nature of the crop with no regular cultivation coupled with the compaction from animal trampling and tractor usage. For these ecosystem services, roots and soil biota play an important role. When sustainable grassland systems are developed it is important to know which effect management measures have on roots, soil biota and the functioning of the soil-plant system. One of the management measures that may contribute to sustainable grassland systems is the introduction of white clover (*Trifolium repens*) with its ability to fix atmospheric N_2 in symbiosis with *Rhizobium* bacteria. However, it is well documented that the root density of white clover is considerably lower than that of grass (Robinson and Jacques 1958; Young *et al.* 1958; Evans 1977; Tisdall and Oades 1979). Since the organic material released by living or decomposing roots stabilizes aggregates directly or indirectly by providing nutrients to micro-organisms, the lower root density could have a direct impact on soil structure maintenance. Robinson and Jacques (1958) measured a lower percentage of stable soil aggregates in white clover than in perennial ryegrass. On the other hand, Sears (1950) and Van Eekeren *et al.* (2005) found a higher earthworm biomass in a grass-clover mixture than in grass-only swards. Earthworms are known for their positive effect on soil structure and water regulation through their burrowing activity and earthworm burrows characteristics (Hoogerkamp *et al.*1983; Clements *et al.* 1991). Mytton *et al.* (1993) found higher drainage rates in white clover than in perennial ryegrass. Altogether this would suggest that with the introduction of white clover in grassland, soil structure maintenance could deteriorate, while water regulation would improve.

In the present field study, we measured the root biomass, the abundance of earthworms and a selection of soil physical parameters in white clover-only, a grass-clover mixture, and grass-only with and without inorganic N fertilizer. Our objectives were (1) to measure the effect of white clover, perennial ryegrass and a grass-clover mixture on the root biomass and abundance of earthworms, and (2) to explore the relevance of changes for the ecosystem services soil structure maintenance and water regulation.

Methods
Sampling site and experimental design
The experiment was established in spring 2004 on a free-draining sandy loam soil (7.2-7.5 % clay (< 2 µm)) in the east of the Netherlands (52º26´N, 6º08´E). Four treatments were established in a completely

randomized block design of six blocks:
GN1 : Grass with inorganic N fertilizer;
GN0 : Grass without N fertilizer;
GCN0 : Grass-clover without N fertilizer;
CN0 : Clover without N fertilizer.
The former production pasture (mainly based on *Lolium perenne*) was killed in March 2004 with 3 L /ha Roundup® Max (Monsanto Company, St Louis, USA), after which the sward was ploughed and prepared for sowing. On 26 April, the different treatments were applied. The seed used was 35 kg *L. perenne* L./ha (cvs. Plenty and Roy) for the grass-only treatments (GN1 and GN0), 30 kg *L. perenne* L. /ha and 5 kg *T. repens* L./ha (cv. Alice) for the treatment GCN0 and 10 kg *T. repens* L./ha for the treatment CN0. In order to get approximately the same quantity and quality (C/N ratio) in the above- and below-ground biomass in GN1 and GCN0, inorganic fertilizer (calcium ammonium nitrate 27%) was applied on GN1 at a rate of 150 kg N/ha. The percentage clover dry matter in 2005 was on average 26% for GCN0 and 75% for CN0.

Soil sampling and analysis
On 16 December 2005, two growing seasons after the start of the experiment, soil samples were taken for determination of root biomass, earthworm biomass, earthworm and earthworm burrow abundance, and soil structure. Three soil cores (0-10 cm, ø 8.5 cm) per plot were taken to determine the root biomass. The soil in the samples was thoroughly rinsed with water, after which the roots were oven-dried at 70 °C and the dry matter of the roots was measured. After drying, the individual samples of roots were bulked together per treatment and analyzed for ash content and total N. Root biomass was expressed as grams of ash-free dry matter (AFDM). Earthworms were sampled in two blocks (20 x 20 x 20 cm) per plot. The blocks were transferred to the laboratory where the earthworms were hand-sorted, counted, weighed and fixed in alcohol prior to identification. Numbers and biomass were expressed per m^2. Before the blocks were sorted for earthworms, in one block per plot the earthworm burrows with a diameter >2 mm were counted on horizontal surfaces (20x20 cm) exposed at 10 cm and 20 cm depth. Bulk density was measured in the 5-10 cm layer below the soil surface, in three undisturbed ring samples containing 100 cm^3 soil. Penetration resistance was measured with a penetrometer (Eijkelkamp, Giesbeek, The Netherlands) with a cone diameter of 2 cm^2 and a 60° apex angle. Cone resistance was recorded per cm of soil depth and expressed as an average value of 6 penetrations per plot in the soil layers of 0-10 cm, 10-20 cm, 20-30 cm. Soil structure was determined in 1 block (20 x 20 x 20 cm) per plot. The soil was divided by visual observation into crumbs, sub-angular blocky elements and angular blocky elements (FAO 2006). These were weighed and expressed as a percentage of total fresh soil weight.

Statistical analysis
The effects of grass-only and fertilization, clover-only, and the mixture of grass-clover on the measured parameters were tested using one-way ANOV, using the GENSTAT statistical software (8^{th} Edition, VSN International, Hemel Hempstead, UK)

Results
CN0 had significantly lower grass root biomass and significantly higher clover root biomass than the other treatments (Table 1). The ranking of treatments in terms of total root biomass was comparable to that of grass root biomass. In terms of the total N in the root biomass, CN0 was significant lower than the other treatments. The C/N ratio in the total root biomass was lowest for CN0 and highest for GN0. GN1 and GCN0 were intermediate. Earthworm abundance was significantly higher in CN0 than in the other treatments (Table 1). CN0 had the highest earthworm biomass, GN1 and GN0 the lowest. Earthworm numbers and biomass were negatively correlated with the C/N ratio of the root biomass (r=-0.59, P=0.002 and r=-0.52, P=0.01, respectively). The number of earthworm burrows at 10 cm depth was significantly higher in CN0 than in the other treatments. At 20 cm depth, the number of earthworm burrows was highest in the two treatments with clover (GCN0 and CN0), but it was not significant different from GN1. The number of burrows at 10 cm and 20 cm depth was positively correlated with the earthworm biomass (r=+0.50, P=0.012 and r=+0.49, P=0.015, respectively).

Bulk density was not significantly different between the treatments. The penetration resistance in all soil layers was lower in clover-only (CN0) than in the grass-only with inorganic N fertilizer (GN1), but this was only statistically significant in the soil layer at 20-30 cm depth. The penetration resistance at 20-30 cm was negatively correlated with earthworm biomass (r=-0.47, P=0.02).The proportion of crumbs was significantly higher in GN0 than CN0 (Table 1). GN1 and GCN0 took an intermediate position. The CN0 had the highest proportion of angular blocky elements. The proportion of crumbs was negatively correlated with clover root biomass (r=-0.53, P=0.008), but no significant correlation was present with grass or total root biomass.

Table 1. Root biomass, earthworm abundance, earthworm burrow number and soil structure in grass with added inorganic N fertilizer (GN1), grass without N fertilizer (GN0), grass-clover without N fertilizer (GCN0) and clover without N fertilizer (CN0).

Parameter	Unit	Treatments				
		GN1	GN0	GCN0	CN0	P-value
Roots biomass 0-10 cm						
Grass	g AFDM/m^2	169a	217a	177a	12b	*<0.001*
Clover	g AFDM/m^2	0c	1c	16b	62a	*<0.001*
Total	g AFDM/m^2	169a	218a	193a	73b	*<0.001*
Total N	g N/m^2	4.0a	4.1a	4.5a	2.6b	*0.043*
C/N		21.0b	26.3a	21.3b	14.2c	*<0.001*
Earthworms						
Total number	n/m^2	322b	326b	359b	480a	*0.002*
Total biomass	g/m^2	82b	76b	110ab	135a	*0.009*
Earthworm burrows						
10 cm depth	n/m^2	58b	67b	138b	225a	*0.002*
20 cm depth	n/m^2	50ab	8b	113a	121a	*0.023*
Bulk density	g/cm^3	1.47	1.42	1.49	1.47	0.098
Penetration resistance						
0-10 cm	mPa	1.48	1.44	1.46	1.39	0.776
10-20 cm	mPa	1.46	1.45	1.40	1.34	0.368
20-30 cm	mPa	2.51a	2.39ab	2.45ab	2.13b	*0.036*
Soil structure 0-10 cm						
Crumb	%	39bc	53a	50ab	32c	*0.006*
Sub-angular	%	13	9	12	5	0.094
Angular	%	47b	38b	38b	62a	*0.009*

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

Discussion

In line with other research (Robinson and Jacques, 1958; Young *et al.* 1958; Evans, 1977; Tisdall and Oades, 1979), the root biomass in clover-only (75% clover in the dry matter in 2005) was less than in grass-only. However, the mixture of grass and clover (26% clover in the dry matter in 2005) had the same root biomass as grass-only. Although the soil structure (measured as proportion of crumbs) was only correlated with clover root biomass and not with grass or total root biomass, the soil structure followed the same pattern; the soil structure in clover-only (measured as a proportion of crumbs) was less developed than in grass-only and the grass-clover mixture. This is in line with other research (Robinson and Jacques, 1958; Tisdall and Oades, 1979) in which perennial ryegrass had a higher soil aggregate stability than white clover-only. Since the grass root mass and the soil structure in the grass-clover mixture were comparable with the grass-only treatments, we suggest that the soil structure of clover mixed with grass is maintained at the same level. Further research on soil aggregate stability is needed for confirmation.

The earthworm biomass was higher (70%) in clover-only (CN0) than in grass-only (GN1 and GN0), with the mixture of grass and clover in an intermediate position. Sears (1950) and Van Eekeren *et al.* (2005) found a higher earthworm biomass in a grass-clover mixture than in grass-only swards. Thus, introduction of clover in a grass sward results in higher earthworm population densities. The negative relationship between the C/N-ratio of the root biomass and the total abundance of earthworms, suggests that the quality of the litter rather than the quantity played a prominent role in the higher abundance of earthworms. Water regulation as an ecosystem service in grasslands is greatly influenced by earthworms (Clements *et al.* 1991; Bouché and Al-Addan 1997). Especially earthworm burrows can increase water infiltration (Edwards and Shipitalo 1998). In our experiment, the numbers of earthworm burrows at 10 and 20 cm depth were highest in clover-only. Furthermore, clover-only showed the lowest penetration resistance at 20-30 cm, suggesting improved water infiltration. These data are consistent with results of Mytton *et al.* (1993), who found that white clover-only drained more rapidly than grass-only. In their research, soil moisture curves indicated a more free-draining structure in clover than in grass due to a higher ratio of macro- to micro-pores (Mytton *et al.* 1993).

For both drainage and soil moisture characteristics, Mytton *et al.* (1993) found that a grass-clover the mixture (> 50% clover in the DM) took an intermediate position between the monocultures of grass and clover. In our research, the mixture of grass-clover (GCN0), with 26% clover in the DM, showed a higher number of earthworm burrows and a lower penetration resistance than grass-only with fertilization (GN1), but differences were not significant. This suggests that a positive effect of clover on water infiltration was not apparent in our grass-clover mixture. With a higher clover percentage in the dry matter this might be different.

Conclusions

The treatment with clover-only had a lower root biomass, a lower C/N-ratio of the roots, a higher abundance of earthworms, a higher number of earthworm burrows, a lower penetration resistance at the 20-30 cm soil layer and a lower proportion of crumbs in the soil, than the other treatments. This confirms the literature that pure clover stimulates the ecosystem services of water regulation, but is less conducive to soil structure maintenance. However, the grass-clover mixture did not differ significantly from the grass treatments, but differed from pure clover in a higher percentage of soil crumbs. We infer that, when clover is introduced in grassland to reduce the reliance on inorganic fertilizer, the mixture of grass and clover maintains the positive impact of grass roots on soil structure but only may show a positive effect of clover-only on water regulation with a higher clover percentage in the dry matter than in our experiment.

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1 – 6 August 2010, Brisbane, Australia. Published on DVD.