Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

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Effects of different crop rotations on controlling clover root rot

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Umeå

Rapport 1:2019

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Nyckelord: växtföljd, klöver, rotröta, foderraps

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Sammanfattning
Vi studerade effekten av växtföljder med olika andel vall och särskilt om förfruktseffekt av grönfoderraps kan minska rotröta i rödklöver. Klöverplantor, ett till tre och ett halvt år gamla, med intakta rötter samlades in från ett långliggande växtföljdsexperiment i Offer i Västernorrland under våren och senhösten 2017. Klöverrotröta graderades enligt DSI (disease severity index) och mängden av patogenen *Fusarium avenaceum* utvärderades med DNA-teknik. Resultaten visade att nivån av infektionen av klöverrotröta direkt beror på åldern av klövern oavsett växtföljd. Trots att det fanns en högre DNA-koncentration av *F. avenaceum* i ettåriga klöver från växtföjden med mer vall fanns det inget sådant samband i äldre klöver. Vi hittade heller ingen stark korrelation mellan klöverrotröta och DNA-koncentration av *F. avenaceum* i klöver. Sammanfattningsvis var de grödor och växtföljder som används i detta långsiktiga växtföljdsexperiment inte effektiva för att kontrollera klöverrotröta. Detta projekt har finansierats av Kungliga Skogs- och Lantbruksakademien.

Abstract
We studied effects of crop rotations with different amounts of grass/clover ley, especially the effect of fodder rape on the development of clover root rot in red clover. Clover plants, one to three and a half years old, with the root-system were collected from a long-term crop rotation experiment in spring and late autumn. Disease severity index (DSI) of clover root rot and the abundance of the clover root rot causal organism *Fusarium avenaceum* was evaluated. The results showed that development of clover root rot clearly depends on the age of clover plants regardless of the cropping system. Even though there was a higher abundance of *F. avenaceum* in one year old clover from crop rotations with more ley, this effect did not persist with increasing age of clover. We did not find any robust correlation between the level of DSI and the abundance of *F. avenaceum* in clover root. In conclusion, the crops and crop rotations used in this long term-experiment were not effective as control of clover root rot and did not increase the longevity of clover in the leys. This project has been financed by The Royal Swedish Academy of Agriculture and Forestry.

Introduction

Red clover (*Trifolium pratense)* is a predominant legume forage crop in Sweden and also an important source of green manure on many farms with organic production. Clover is grown throughout the country and nearly 40% of agricultural land in Sweden is covered by clover/grass (Statistics Sweden, Statistical Yearbook of Sweden 2008). It is mainly grown in association with grasses for production of forage as animal fodder. However, farmers have great interest in clover due to its high nutritional value and ability to improve soil structure and fertility (Ericson, 2005). Clover belongs to the leguminous family and has great ability to fix nitrogen (N) through a symbiosis with bacteria and annually fix 22-85 kg N/ha in Sweden (Carlsson et al., 2009). Usually, red clover is high yielding (e.g. Wiersma et al., 1998), but when used as perennial leys for silage, soil-borne pathogens often reduce stand longevity (Rufelt 1986; Skipp, 1986). The etiology of soil-borne fungal pathogens is dominated by the genera *Fusarium, Cylindrocarpon, Phoma, and Pythium* (Lager and Gerhardson, 2002). These pathogens can initiate several diseases in clover and one of the most damaging disease is clover root rot (Lager and Wallenhammar, 2003) caused by different *Fusarium* spp. (Rufelt, 1979) and *Cylindrocarpon destructans* (Öhberg, 2008). In addition, some species of *Fusarium* can produce mycotoxins, which are toxic to humans and animals through consumption of contaminated food and feed (D'Mello et al., 1999). A nationwide survey in Sweden indicated that the occurrence of *Fusarium* root rot of clover is very common (Rufelt, 1979). Resistant cultivars to *Fusarium* spp. are not suitable in all Swedish regions and the application of fungicide is restricted (Öhberg, 2008). Therefore, it is necessary to find alternative approaches to control clover root rot and diversified crop rotation could be one option.

The practice of crop rotation within agro-ecosystems aims to reduce soil erosion, properly utilize soil nutrients, improve soil structure (Carroll et al., 1997; Triberti et al., 2016) and to control pest and pathogens (Hossain et al., 2012, 2014, 2015). Crop rotations including perennial leys allows for increased soil organic matter (SOM) by decaying incorporated biomass with active microorganisms and lead to aggregate nutrient retention and utilization (Baldwin, 2006). In addition, some rotational crops release root exudates and other secondary chemicals (Hossain et al., 2015) within root-soil environment and alleviate allelopathy (Kirkegaard et al. 2000) thus controlling pest and pathogens (Hossain et al., 2012 and 2014). *Brassica spp.* have an allelopathic effect against soil microbes (Hossain et al. 2015). Muehlchen et al. (1990) showed that incorporation of *B. napus* plant tissue can reduce the development of pea root rot in a subsequent pea crop. Recently, Hossain et al. (2015) showed that white mustard (*Sinapis alba*) significantly reduced Aphanomyces pea root rot disease severity, when *S. alba* was grown in *A. euteiches* infested soil for more than two months.

In this pilot study, our objective was to find the residual effects of rotational crops on the development of clover root rot in perennial forage, especially the effects of fodder rape. The hypothesis is that diversified crop rotations including fodder rape, *Brassica napus,* contribute to reduced disease severity of root rot in clover and increase the clover content by improving the longevity of the clover in clover/grass leys. In order to achieve our objective, we collected clover samples with root-systems twice from a long-term experiment and evaluated disease severity of clover root rot and the abundance of *Fusarium avenaceum*, one of the causal organisms of clover root rot.

Materials and methods

Long-term experiment

Clover plants were collected from a long-term experiment at Offer, Västernorrlands län, Sweden (Figure 1). This experiment started in 1956 and the crops were changed in 1987 (Zhou et al., 2018). The main objectives of this experiment are to investigate how different crop rotations affect soil structure, fertility and also how crops residues effect the growth and development of following crops. In this experiment, different crops typical for the area such as fodder rape (*Brassica napus*), barley (*Hordeum vulgare*), red clover (*Trifolium pratense.)* and potatoes (*Solanum tuberosum*) and grass (Poaceae spp.) are grown as rotational crops in six consecutive years.

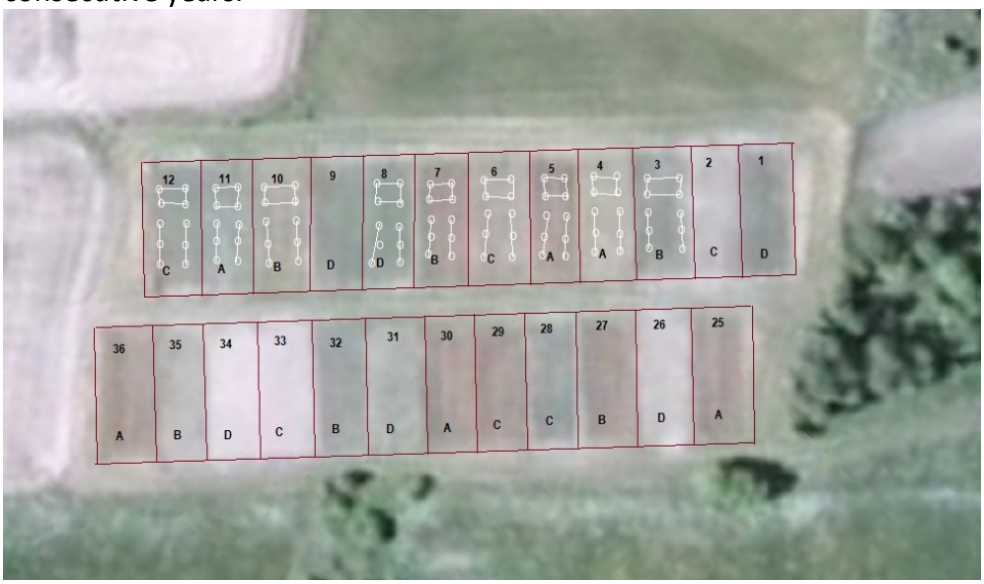

Figure 1, Field location (Offer, Västernorrland Län, Sweden), plots alignment (1-36), cropping systems (A-D), clover roots sampling method (white circular) and samples pooled together for DNA extraction (white line or rectangular).

There are four different crop sequences with grass/clover leys that last from one to six years in the different crop sequences (Table 1). Two of these sequences also include a Brassica inter-crop (fodder rape/ peas/ barley). Each sequence is represented on six plots and each year all crops in the sequence are represented on one plot (Figure 1). The design is a randomized block design.

Table 1. Crop sequences

year	A	B	C	D
1	Barley under-sown ley	Barley under-sown ley	Barley under-sown ley	Barley under-sown ley
2	Clover/grass year I	Clover/grass year I	Clover/grass year I	Clover/grass year I
3	Clover/grass year II	Clover/grass year II	Clover/grass year II	Barley
4	Clover/grass year III	Clover/grass year III	Barley	Potatoes
5	Clover/grass year IV	Barley	Potatoes	Barley
6	Clover/grass year V	Brassica/pea/barley	Brassica/pea/barley	Potatoes

The crop sequences A and B are intended to represent two pronounced fodder crop rotations. Crop sequence C is partly aimed for production of directly sold crops. Plant sequence D is focused on sold crops and one green manure grass/clover crop.

Incidence of clover root rot

Ten clover plants including the main root-system were collected from both sides (5+5) of each plot (Figure 1), in May and November in 2017. Disease severity index (DSI) was registered as the degree of internal (vascular) root symptoms and external root symptoms (lesions and discoloration) according to Rufelt (1979). All roots were separated from the shoot at the hypocotyl and were washed with running tap water and examined for external lesions and discoloration. After that, all washed roots were cut longitudinally to visually examine the internal disease severity and finally overall DSI was set by evaluating both external and internal disease symptoms of each clover plant root system. Each individual plant was assigned one of five scores for disease severity: 0 = healthy plant; 1 = root slightly discolored; 2 = root extensively discolored and partly rotted; 3 = root extensively discolored and rotted; 4 = root completely rotted or plant dead (Figure 2). After determining DSI, all samples were stored at -20 ^{0}C for analyzing the abundance of one of the causal organisms (*F. avenaceum*) of clover root rot.

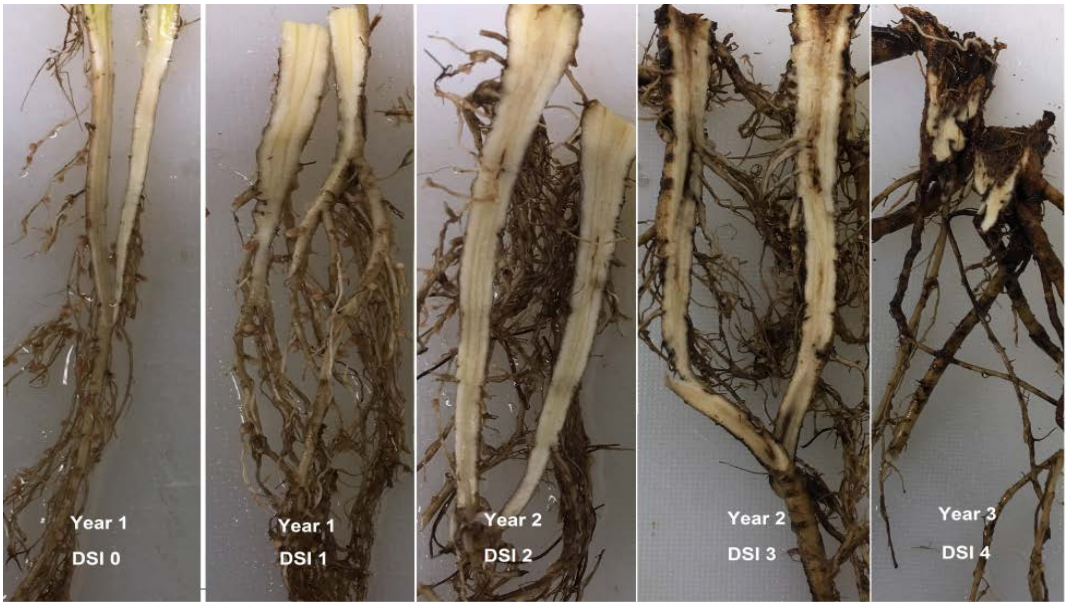

Figure 2. Disease severity index (DSI) of clover root rot with internal and external root rot symptoms (lesions and discoloration) and the age of clover plants. Disease severity index (DSI): 0 = healthy plant; 1 = root slightly discolored; 2 = root extensively discolored and partly rotted; 3 = root extensively discolored and rotted; 4 = root completely rotted or plant dead (Fig. 2). Photo, DSI (1-4) Shakhawat Hossain.

Abundance of *F. avenaceum* in clover root.

Fungal isolate: Fresh fungal isolate of *Fusarium avenaceum* (isolate, **EVP3**) was used for standard curves. This isolate was obtained from the Dept. of Crop Production Ecology (VPE), Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

DNA extraction from fungal isolate: The isolate *F. avenaceum* was grown on PDA medium for 1 week prior to DNA extraction. Mycelium was scraped off the agar plate using a spatula and homogenized in liquid $N_{2.}$ DNA was extracted from 500 mg of homogenized mycelium by using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The concentration 3 ng/µl of DNA from the pure fungal isolate was used for the standard curve (Nicolaisen et al., 2009) in five-fold dilution series of the isolate.

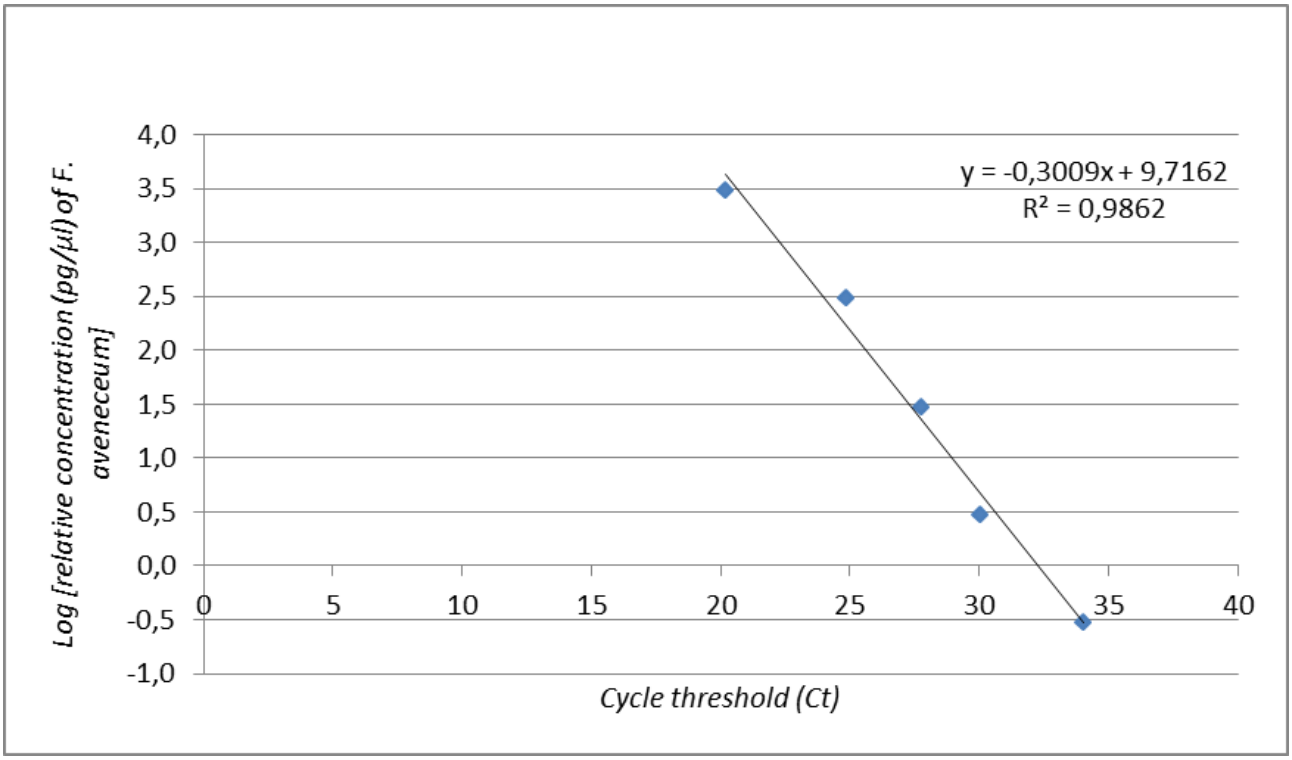

Figure 3. Standard curve made from five dilution factors of extracted DNA (3 ng/µl) from *F. avenaceum* mycelium.

DNA extraction from clover roots: Root samples were pooled together according to Figure 1 and ground in liquid N_2 by using mortar and pestle. DNA extraction procedure was following the manufacturers recommendations (DNeasy Plant Mini Kit,QIAGEN, Hilden, Germany) and this procedure was conducted at Umeå Plant Science Centre (UPSC), Umeå, Sweden.

Primers: Primer "Fave574 fwd TATGTTGTCACTGTCTCACACCACC and Fave627 rev AGAGGGATGTTAGCATGATGAAG" used for identify and quantification of *F. avenaceum* (Nicolaisen et al., 2009 ref). The specificity of the primer was already tested on DNA from Fusarium using real-time PCR (Nicolaisen et al., 2009). The primers were also used to estimate the DNA yield from each sample and to derive a normalized measurement of infection which was calculated as pg fungal DNA per µl plant DNA.

Real-time PCR: Real-time PCR was carried out in a total volume of 12.5 µl consisting of 6.25 µl 2x SYBR Green PCR Master Mix (Applied Biosystems), 250 nM of each primer, 0.5 µg/µl bovine serum albumin (BSA) and 2.5 µl template DNA. PCR reactions were performed in duplicate on all samples. Genomic DNA from root samples and pure culture were adjusted to 3 ng/µl (Table 2).

PCRs were performed on a Roche Light Cycler 480 using the following cycling protocol: 2 min at 50 °C; 95 °C 10 min; 40 cycles of 95 °C for 15 s and 62 °C for 1 min followed by dissociation curve analysis at 60 to 95 °C.

Quantification of Fusarium in clover root: The amount of fungal DNA was calculated from cycle threshold (Ct) values using the standard curve, and these values were normalized with the estimated amount of root DNA.

Statistical analysis: The correlations between disease severity index (DSI) or DNA abundance of *F. avenaceum* (Figure 5) in clover roots, with cropping system, sampling time and age of clover plants were tested with a $P \leq 0.05$ significance level by using R version 3.4.3 (The R Foundation, 2017).

Results

Development of clover root rot

The development of clover root rot DSI clearly depends on the increasing age ($P < 0.001$) of the clover plants and the correlation between DSI and the age of clover is high (figure 4) , where one year old clover crop had lower DSI than two or three years aged clover (Table 2). The corresponding mean DSI was 1.3, 1.4, 2.4, 3.4, 3.7 and 3.6 respectively in clover age 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 years (Figure 4). Disease severity index in 1 year clover was lower in cropping system D compare to other cropping systems, but since there was only one plot with clover in cropping system D we cannot tell if this was just by chance or not. The mean DSI in November 2017 was higher than DSI in May the same year and accelerated mostly during the second year of clover growth (Table 2).

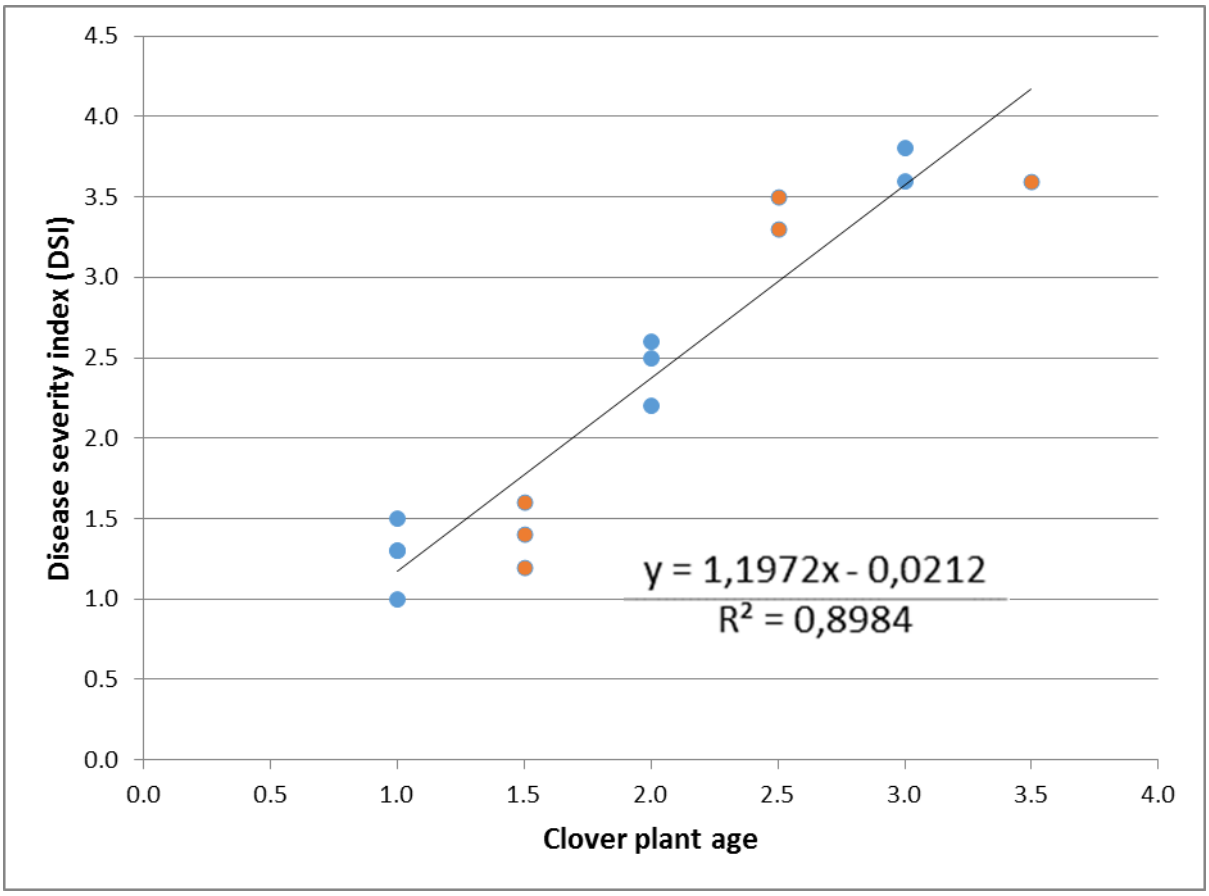

Figure 4. The development of DSI in different clover of different age was registered from different cropping systems in a long-term experiment. The scale for disease severity index (DSI): 0 = healthy plant; 1 = root slightly discolored; 2 = root extensively discolored and partly rotted; 3 = root extensively discolored and rotted; 4 = root completely rotted or dead plant. Blue dots are from the May sampling and red dots are from the November sampling.

Table 2. Clover root rot symptoms, concentration (ng/µl) of clover root DNA and abundance (pg/µl) of *Fusarium avenaceum* in infected clover root tissues, estimated from a long-term experiment. The scale for disease severity index (DSI): 0 = healthy plant; 1 = root slightly discoloured; 2 = root extensively discoloured and partly rotted; 3 = root extensively discoloured and rotted; 4 = root completely rotted or plant dead.

Plot	Sampling time (2017)	Plant age (year)	Cropping system[1]	DSI	SE	Plant DNA (ng/µl)	SE	Adjusted plant DNA (ng/µl)	Plant fungus DNA (pg/µl)	SE
P11	May	1	A	1,3	0,15	27,17	2,96	3	0,33	0,13
P11	Nov	1,5	A	1,2	0,13	20,57	0,29	3	0,83	0,54
P3	May	1	B	1,3	0,15	29,67	0,60	3	0,11	0,03
P3	Nov	1,5	B	1,4	0,16	16,70	0,35	3	0,81	0,36
P12	May	1	C	1,5	0,17	31,13	1,44	3	0,08	0,03
P12	Nov	1,5	C	1,6	0,16	20,50	3,16	3	0,94	0,23
P8	May	1	D	1,0	0,00	34,03	1,67	3	0,06	0,00
P4	May	2	A	2,6	0,16	28,83	2,50	3	0,70	0,10
P4	Nov	2,5	A	3,5	0,22	35,27	2,79	3	0,65	0,17
P10	May	2	B	2,5	0,22	27,90	1,59	3	1,37	0,82
P10	Nov	2,5	B	3,3	0,21	32,77	1,43	3	0,61	0,12
P6	May	2	C	2,2	0,13	26,57	1,96	3	0,66	0,27
P5	May	3	A	3,8	0,13	28,63	0,63	3	0,45	0,20
P5	Nov	3,5	A	3,6	0,22	33,80	5,93	3	1,14	0,40
P7	May	3	B	3,6	0,22	28,70	0,64	3	0,51	0,11

SE, Standard error

1, Cropping system (A-D) according to table 1

Abundance of *F. avenaceum* in clover root

The level of DNA concentrations (pg/µl) of *F. avenaceum* in clover roots varied over the age of the clover plant, plant sampling time and also cropping system (Table 2).The DNA concentration of *F. avenaceum* increased ($P < 0.001$) with the growing age of clover, where DNA concentration was lower in first year clover than older clover, especially in 3.5 years old clover (Table 2). The corresponding mean DNA concentration was lower in sampling time May 2017 than in sampling time November in the same year. At the May sampling of one-year old clover roots there was a trend (p=0,056, r=0.944) towards higher DNA concentration of *F. avenaceum* with increasing number of years with ley within each crop rotation (Figure 5). However, this did not persist in the later sampling and was not seen in older grass/clover leys.

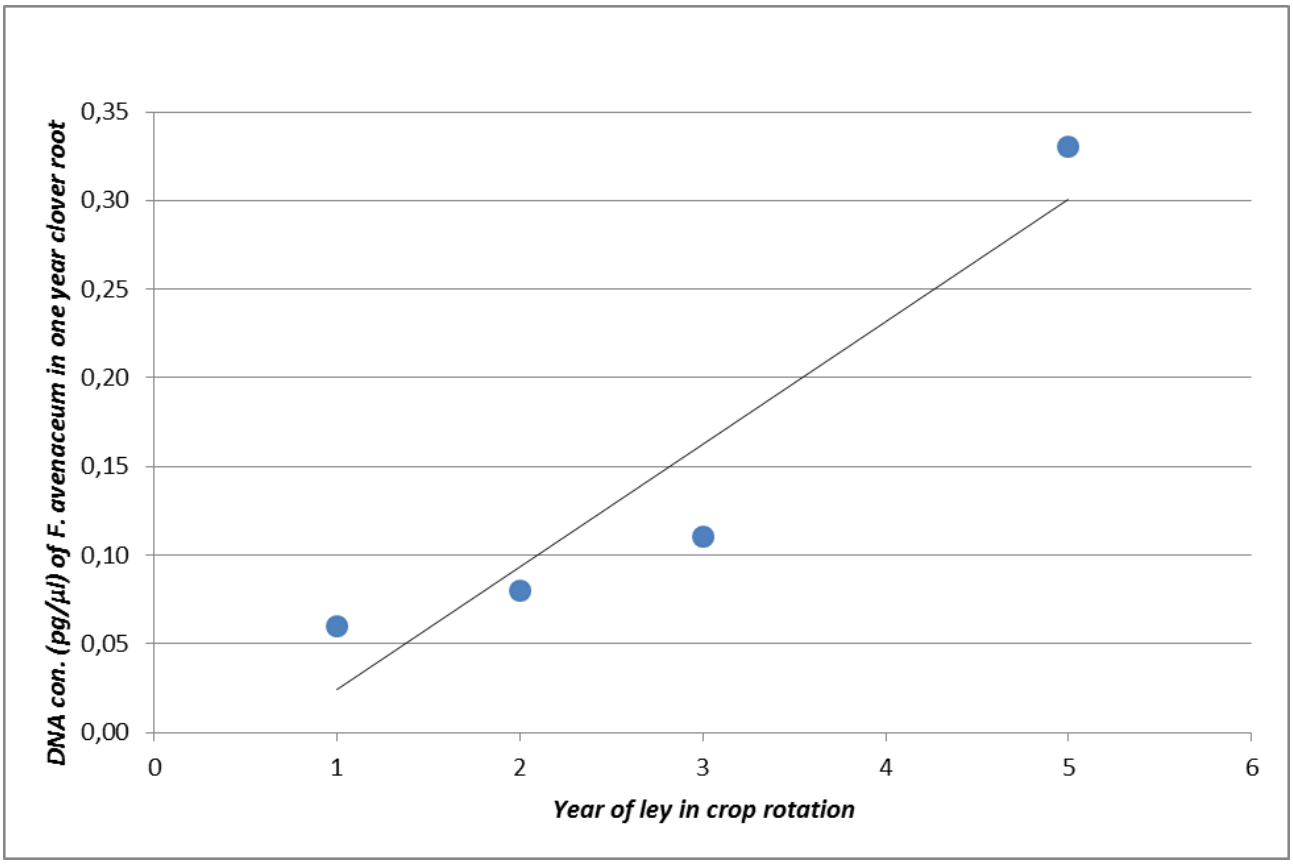

Figure 5. The DNA concentration (pg/µl) of *F. avenaceum* registered in one-year old clover roots from different cropping systems (A-D).

Discussion

In this study, overall results showed that development of clover root rot DSI, increased with increasing age of the clover crop, regardless of the cropping system (Table 2). Clover root rot disease can be initiated by several pathogenic organisms including *F. avenaceum*, through a complex process, according to Lager and Gerhardson (2002). The *Fusarium* genus has a wide range of host plants and cause diseases in many different crops including cereals and forage crops (Leslie et al., 1990; McMullen et al., 1997). Due to this capability, *Fusarium* spp. are usually not affected by different cropping systems (Leslie et al., 1990; Marburger et al., 2015). In this study, grass /clover leys, which followed after a mixture of fodder rape (*Brassica napus*), pea (*Pisum sativum*) and barley (*Hordeum vulgare*) in cropping systems B and C, unexpectedly, did not show any residual effect from *B. napus* on the development of root rot in the following clover crop (Table 2). Thus our hypothesis was not confirmed. One reason is the limited number of samples. Another reason could be that any effect of gucosinolates (GSLs) in *B. napus* residues had diminished during the winter season, between the crops, since the residual effect from *B. napus* diminishes over time (Hossain et al., 2012). Hossain et al. (2014) showed that volatile hydrolyzed products of GSLs from Brassicaceae tissues disappear within an hour, when the hydrolyzing process going on in the presence of water in open conditions, exposed to air. Also, different Brassicaceae species are dominated by different GSLs (Matthiessen and Shackleton 2005) and all are not efficient against fungal pathogens. In addition, *F. avenaceum* can infect other crops such as preceding intercropped pea in rotation B and C (Lager and Gerhardson 2002) and thus survive periods without host clover.

There was an indication that the level of clover root rot (DSI) in one-year old clover was lower in cropping system D compared to other cropping systems (Table 2). In addition, DNA concentration of *F. avenaceum* in one-year old clover roots in the cropping system D was also lower compared to other cropping systems (Fig. 5). This result indicated that, in a crop rotation with grass/clover as a green manure crop, where clover was grown for a shorter time (two growing seasons), clover root rot never got enough time to spread all over the root system and the causing organisms did not have time to multiply.

The poor correlation between clover root rot (DSI) and *F. avenaceum* DNA results indicate that there are other pathogens directly or indirectly involved in the disease process, which has been suggested by others (Lager and Gerhardson, 2002; Lager and Wallenhammar, 2003: Öhberg, 2008).

Conclusions

We found no evidence that long lasting leys (five years) lead to more clover root rot or more *F. avenaceum* in clover roots than 2-3 year old leys. Fodder rape intercropped with pea and barley did not have any detectable disease suppression effects on clover root rot.

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