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REGULAR ARTICLE

Brassicaceae cover crops reduce Aphanomyces pea root rot without suppressing genetic potential of microbial nitrogen cycling

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Abstract
Aims Brassicaceae cover crops can be used to suppress soil-borne pathogens. The aim was to investigate the effect of different brassicas with different glucosinolate profiles on the development of Aphanomyces pea root rot in subsequent pea plants, and the genetic potential of free-living N_2-fixing bacteria and ammonia oxidising bacteria (AOB) and archaea (AOA) performing key soil ecosystem services.
Methods The Brassicaceae species *Brassica juncea* and *Sinapis alba* and non-Brassicaceae species *Secale cereale* were grown for 11-weeks in *Aphanomyces euteiches* infested soil at low and high nitrogen (N) fertiliser doses. After removing both shoots and roots of the cover crops, peas were grown as a bioassay to evaluate Aphanomyces pea root rot development. Soil was sampled before harvesting the cover crops and at the end of the bioassay. Volatile compounds were collected in the root-soil environment before harvesting the Brassicaceae cover crops to determine the concentration of isothiocyanates. The abundance of genes involved in N_2-fixing bacteria and ammonia oxidation in AOA and AOB were assessed.
Results Pea root rot disease severity was reduced in Brassicaceae grown soil at the high N fertiliser dose. This was associated with increased growth of the cover crops. The growth of Brassicaceae did not suppress the abundance of N-cycling microbial communities, but rather increased the AOB at the end of the bioassay, most likely due to increased N availability. The disease suppressive effect was higher with *S. alba* than with *B. juncea*, and this coincided with a more diverse composition and higher concentration of aliphatic ITCs released from *S. alba* roots. Fewer nodules were formed after the Brassicaceae crops, especially *Sinapis alba*.
Conclusions Brassicaceae cover crops, particularly *S. alba,* can be used to control soil-borne pathogens without major side effects on the genetic potential of beneficial soil microorganisms involved in N cycling. However, less nodule formation after brassicas indicates an effect on rhizobium activity.

Keywords Glucosinolates · Isothiocyanates · *Aphanomyces euteiches* · *Brassica juncea* · *Sinapis alba* · Nitrogen fertlisation · Biofumigation

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Introduction

The persistent and globally distributed soil-borne pathogen *Aphanomyces euteiches* is an oomycete causing root rot in legumes, which is a severe problem in commercial pea (*Pisum sativum* L.) production (Papavizas and Ayres 1974; Gaulin et al. 2007; Persson 2008). Pea root rot is difficult to control without long intervals (6–8 years) between pea crops in the crop rotation. Biofumigation using plants from the Brassicaceae family is however of interest since many Brassicaceae species produce sulphur-containing secondary metabolites known as glucosinolates (GSLs) (Sang et al. 1984; Fahey et al. 2001; Hossain et al. 2012). When the GSLs are hydrolysed by the endogenous enzyme myrosinase, volatile isothiocyanates (ITCs), thiocyanates and water-soluble nitriles and epithionitriles are formed (Brown and Morra 1997; Kiddle et al. 2001). These products are toxic to soil-borne pathogens (Kirkegaard et al. 2000; Potter et al. 2000; van Dam et al. 2009), but the suppressive effect depends on their chemical composition (Smolinska et al. 2003; Matthiessen and Shackleton 2005) and concentration (Angus et al. 1994; Sarwar et al. 1998; Hossain et al. 2014). For the pea root rot pathogen *A. euteiches*, in-vitro experiments have demonstrated reduced hyphal growth caused by volatiles from *Brassica napus* rapeseed meal (cv. Dwarf Essex) (Dandurand et al. 2000) and *B. juncea* (cv. Corron) shoot tissue (Hossain et al. 2014). Several field studies have shown that incorporation of *B. napus* or *Sinapis alba* (white mustard) plant tissue can reduce the incidence or development of root rot in subsequent pea crops (Chan and Close 1987; Muehlchen et al. 1990).

Most examples of biofumigation using brassicas are based on macerating the plants and incorporating them into the soil to achieve a sudden boost of toxic volatiles (Angus et al. 1994; Kirkegaard et al. 2000). Less is known about the effects of living and growing roots of brassicas on *A. euteiches*. A few studies have shown that intact Brassicaceae roots have an impact on fungal and bacterial communities (Rumberger and Marshner 2004; Bressan et al. 2009), and a negative effect on fungal spore germination (Schreiner and Koide 1993). Moreover, the amount of GSLs is usually higher in root than shoot tissue (Rosa 1997; van Dam et al. 2009). Borek et al. (1996) found a higher concentration of the myrosinase enzyme in rhizospheric soil of brassicas than in non-rhizospheric soil, and demonstrated that this enzyme actively hydrolysed GSLs.

Since the GSL hydrolysis products can suppress plant pathogens, there is concern over their toxic effect on beneficial organisms, including the rhizobium bacteria that are essential for pea root nodule formation for the fixation of atmospheric di-nitrogen gas (N_2) (Kirkegaard et al. 1999). Peas had fewer root nodules when Brassicaceae shoot tissues had been incorporated before sowing (Muehlchen et al. 1990), suggesting that GSL hydrolysis products inhibited bacteria essential for nodule formation, and the rhizobial community associated with living Brassicaceae roots was significantly influenced by changes in GSL root profiles (Bressan et al. 2009). Other soil microorganisms related to the cycling of N and their functions may be affected by GSL hydrolysis products. Bending and Lincoln (2000) found that synthetic ITCs inhibited nitrification activity and decreased growth of nitrifying bacteria, especially the ammonia oxidisers that perform the first step in the nitrification process. These organisms are known to be sensitive to a range of environmental disturbances and have therefore been used as indicator organisms for different types of soil perturbation (Wessén and Hallin 2011).

The aim of the current study was to compare two Brassicaceae species used as cover crops and to determine whether the hydrolysis products of GSLs from the intact growing roots reduce the development of Aphanomyces pea root rot in subsequent peas without affecting beneficial soil microorganisms. Our hypotheses were (i) that growing Brassicaceae crops reduce the development of Aphanomyces pea root rot in subsequent pea plants and (ii) reduce the growth of the soil N_2-fixing and ammonia oxidising microorganisms, thereby decreasing the abundance of these organisms, and (iii) that the effect is greater the better the Brassicaceae plants grow. Further, we predict that the suppressive effects are linked to an increase in ITCs in the soil. This was tested by growing the Brassicaceae species *S. alba* and *B. juncea,* and the non-GSL containing plant *Secale cereale* in *A. euteiches*-infested field soil under two N-fertilisation levels to determine the role of different growth intensities. The effect on the development of Aphanomyces pea root rot was evaluated using a bioassay with pea plants growing in the soil after removal of the cover crops. To evaluate potential negative side-effects on the soil microbial community, we investigated effects on the genetic potential of free-living N_2-fixing bacteria and ammonia oxidising bacteria (AOB) and archaea (AOA) by quantifying genes of

key enzymes involved in N_2 fixation (*nifH*) and ammonia oxidation (*amoA*).

Materials and methods

Pathogen strain

Aphanomyces euteiches strain 5035:8B was used in all experiments (obtained from F. Heyman, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden). The strain was maintained on corn meal agar (CMA) (Oxoid Ltd., UK) at 6 °C and was used for soil inoculation, using a method described by Schneider (1978) modified by Persson et al. (1999). In brief, *A. euteiches* was cultured in oat meal broth for 4 weeks, the mycelium mat of the culture was homogenised, and the oospore concentration quantified, mixed with talcum powder (VWR International) and dried. The dried inoculum material was sieved through a 1-mm mesh net and stored at 4 °C until use.

Soil sampling, experimental set-up, incidence of pea root rot and nodule formation

Soil was collected from an agricultural field in Giresta, Enköping, Sweden, sieved through a 6-mm mesh and stored at 4 °C at a soil moisture content of 21 %. The soil consisted of 46 % clay, 48 % silt, 2 % sand and 4 % organic matter, and the pH (H_2O) was 7.7. The absence of *A. euteiches* was confirmed by growing peas in freshly collected field soil for 4-weeks and assessing root rot development according to Parke et al. (1991).

At the start of the experiment, the soil was inoculated with the pathogen (450 oospores g^{-1} soil) and *Brassica juncea* (cv. Corron), *Sinapis alba* (cv. Architect) and *Secale cereale* (cv. Amilo) were sown separately in 8 experimental pots (5 l). Half of the pots with a cover crop were fertilised with a high N dose (336 kg N ha^{-1} soil) and half with a lower dose (168 kg N ha^{-1} soil). The high level was estimated to be enough for the fastest possible growth rate calculated from a previous greenhouse experiment and the lower level was half that amount. As controls, four replicates each of unfertilised soil and soil fertilised with the different N doses were used. All pots were incubated in the greenhouse (day-night period of 14:10 h and temperature 19:14±2 °C) for 11 weeks. Soil moisture was adjusted daily by checking the weight loss of each pot and water was added with some surplus to avoid possible NO_3^- accumulation. A pre-prepared liquid nutrient solution (1 M $(NH_4)_2SO_4$) was used as fertiliser and applied six times with increasing doses for application 1 to 3 and decreasing doses for applications 4 to 6. The nutrient application was made between 10 and 58 days after sowing the cover crops.

All cover crops were harvested when *S. alba* and *B. juncea* reached flowering stage 11 weeks after sowing, and the shoot tissue was discarded. Cover crop roots were removed from the soil by sieving, and the soil was returned to the original pots to be used in a bioassay. Water was added to the soil and the pots were left in the greenhouse for 2 days to allow any volatiles produced to evaporate, since we wanted to avoid any direct effect of ITCs in the bioassay. Eight pea seeds were then sown in each pot and incubated in the greenhouse for 4 weeks (day-night period 14:10 h and temperature 24:19±2 °C). Soil moisture was adjusted daily by checking the weight loss of each pot and adding water accordingly to obtain optimal infection conditions. Germination of the pea seeds was estimated as percentage of emerged plants.

At the end of the 4-week bioassay, each pot was assigned a DSI (Disease Severity Index) value based on the mean pea root rot symptoms of the individual pea plants in that pot, according to Parke et al. (1991). Each individual plant was assigned one of five scores for disease severity: 0 %=healthy plant; 25 %=root slightly discoloured; 50 %=root extensively discoloured but not shrunken; 75 %=root extensively discoloured and shrunken; 100 %=root partly or completely rotted or plant dead. In addition, pea root nodule formation was ranked based on the observation of all pea plants in each treatment. Each treatment was scored using a scale ranging from 1 to 5 with 1=low nodule formation and 5=high nodule formation.

One week before harvesting the cover crops, 2 g of soil was collected as five sub-samples taken randomly at a depth of 5 cm in each pot. The same procedure was used for the second soil sampling, which was carried out when harvesting the pea seedlings following the 4-week bioassay. The soil samples were stored at −80 °C prior to DNA extraction.

DNA extraction and quantification of *amoA* and *nifH* genes

DNA was extracted from 0.5 g soil sample using the FastDNA® Spin Kit for soil and the FastPrep®

Instrument (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR of *amoA* genes coding for the ammonia monooxygenase enzyme required for NH_3 oxidation was performed to estimate the genetic potential of ammonia oxidation by the ammonia oxidising bacteria (AOB) and ammonia oxidising archaea (AOA). To quantify the genetic potential of the free-living N_2-fixing bacterial community, the gene *nifH* coding for the iron (Fe) part of the nitrogenase enzyme that reduces N_2 to NH_4^+ was targeted. The primer pairs used were *amoA*1F (5′-GGGGTTTCTACTGGTGGT-3′) and *amoA*2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) (Rotthauwe et al. 1997) for bacterial *amoA* (AOB), crenAmoA23F (5′-ATGGTCTGGCTWAGACG-3′) and crenAmoA616R (5′-GCCATCCATCTGTATGTC CA-3′) (Tourna et al. 2008) for archaeal *amoA* (AOA) and Po1F (5′-TGCGAYCCSAARGCBGACTC-3′) and Po1R (5′-ATBGCCATCATYTCRCCGGA-3′) (Poly et al. 2001) for the *nifH* gene (N_2 fixing bacteria). The quantifications were performed in a total volume of 20 μl using the DyNAmo ™ Flash SYBR® Green qPCR kit (Finnzymes, Vantaa, Finland), 0.50 μM of each primer, 0.1 % BSA and 10 ng soil DNA, using the CFX 96 ™ Real-Time System (Bio-Rad Laboratories, Inc, Hercules, CA). Thermal cycling conditions consisted of an initial enzyme activation step at 95 °C for 15 min followed by 35 cycles of 15 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and a final step of 30 s at 80 °C at which fluorescence was acquired to avoid possible interactions with primer dimers and unspecific PCR products. The reactions were finished with a melting curve starting at 60 °C with an increase of 0.5 °C per 5 s up to 95 °C to verify amplicon specificity in addition to checking the amplicons on 1 % agarose gels. Two independent reactions were performed for each sample. Standard curves were obtained using serial dilutions (r^2=1.00 for all curves) of linearised plasmids (pGEM-T Easy; Promega, Madison WI USA) containing cloned fragments of the genes. Bacterial and archaeal *amoA* genes fragments were amplified from a soil sample prior to cloning, whereas the *nifH* fragments were amplified from *Frankia alni* ACN14a. In all three cases, the same primers as in the qPCR assays were used. The PCR runs had an efficiency of 88 % (archaeal *amoA*) and 94 % (bacterial *amoA* and *nifH*). Control samples without templates resulted in undetectable values. Inhibitory effects were tested by running each of the samples together with a known amount of a circular plasmid (pGEM-T Easy) using the plasmid-specific primers T7 and Sp6, and the cycle thresholds (C_t values) obtained were not significantly different from those obtained when amplifying the plasmid in water.

Soil available nitrogen

To determine available ammonium (NH_4^+) and nitrate (NO_3^-) in the soil, 50 g of soil was collected from each experimental pot with the low N dose and the unfertilised control before (first) and after (second) the bioassay and stored at −20 °C. Soil available N-NH_4^+ and N-NO_3^- were extracted with 2 M KCl and determined colorimetrically by flow injection analysis using TRAACS 800 (Bran+Luebbe, Germany).

Analysis of isothiocyanates (ITCs) in the soil

Volatile compounds were collected from the growing roots of Brassicaceae plants after 11 weeks of growth before harvesting. A 10-mm hole was made in the pots 5 cm below the soil surface. A glass tube was inserted in the hole, and into this was inserted a glass liner containing Tenax TA (50 mg 60/80 mesh, Supelco, Bellefont, USA). Air was pulled out through the liner/Tenax at 350 ml min^{-1}. Volatile compounds in the root-soil environment were trapped for periods of 24 h at 24±2 °C under artificial light conditions. Collected volatiles were analysed by gas chromatography (GC) as described by Hossain et al. (2014), using decane (50 ng) as an internal standard for quantification. For tentative compound identification, volatile samples were collected as described above and analysed by coupled GC-mass spectrometry as described by Hossain et al. (2014). Compounds were identified by comparison against a commercially available library (National Institute of Standards and Technology, NIST 08, USA) and by comparison of mass spectra and retention indices with commercially available authentic standards (Sigma-Aldrich AB, Sweden). Standards were unavailable for two compounds, but the retention index (Kovats Index, KI) of the substances could be matched with previously published KIs for the compounds on a HP-1 column.

Statistical analysis

The *nifH* and *amoA* gene copy numbers, DSI values and the soil available N-NH_4^+ and N-NO_3^- were log-10 transformed to equalise variation. The data obtained

from the first soil sampling occasion (low N dose) was used to test the effects of the cover crops against a fertilised and unfertilised control on the variables AOA, AOB and N_2-fixing bacteria, before removing the cover crops. Data from both soil sampling occasions (low N dose) were used to test the effect of sampling time and cover crop treatment on soil mineral N, AOA, AOB and N_2-fixing bacteria. Data from the second soil sampling occasion (both low and high N doses) were used to test the effect of N level and cover crop on AOA, AOB, N_2-fixing bacteria and DSI at the end of the bioassay. Data were fitted in linear models and mean values of the variables in the different cover crop treatments were compared using the Honestly Significant Difference (HSD), Tukey's test with $P<0.05$ significance limit. The unfertilised control treatment was excluded when both N doses were included in the analyses.

To assess whether soils within the different treatments share microbiological properties after the pea growth assay, the variation in abundances of *amoA* genes from AOA and AOB and *nifH* from N_2-fixing bacteria (*nifH*) in addition to DSI-values were compared using principal component analysis (PCA) with a correlation matrix. Difference between variations was examined by taking the sample scores of the ordination diagrams. Thus we focused on inter-sample distances on the significant axes determined by Monte Carlo permutation tests and the rank of each axis was determined by its eigenvalue (λ).

All statistical analyses were performed using the "R" environment (version 2.15.2, The R Foundation, 2013). The PCA analysis was performed using the "FactoMineR" package.

Results

Development of Aphanomyces pea root rot

After growing *S. alba* cover crop for 11 weeks in Aphanomyces-infested soil at high nutrient dose, the pea bioassay revealed significantly lower pea root rot DSI in the Brassicaceae cover crop treatments compared to other treatments (Fig. 1). Growth of *B. juncea* at the high N level resulted in a lower DSI than in the control treatment, but the effect was not significantly different from that of *S. cereale* or the N-fertilised control. No effects of cover crops were found at the low N dose. As hypothesized, the DSI-values were lower after the high N dose than after the low dose ($P<0.001$) across all cover crop treatments, and there was a significant interaction ($P<0.002$) between treatment and N-fertilisation, indicating a larger effect of N with Brassicaceae cover crops, especially *S. alba*, than with *S. cereale* or the fertilised control.

Nodule formation

Eleven weeks of *S. cereale* growth enhanced nodule formation of subsequent peas compared to other treatments (Table 1). Growth of Brassicaceae cover crops, especially *S. alba* resulted in less nodule formation than in the other treatments. Moreover, there were fewer nodules on the pea roots after the high N dose than after the low N dose.

Abundance of genes from N-cycling microbial communities

Treatments with brassicas did not suppress the abundance of *amoA* or *nifH* genes from the ammonia-oxidising microorganisms or the free-living N_2-fixing bacteria (Table 2). The gene copy numbers ranged from 5.0×10^7 to 1.0×10^8 for the AOA, 3.5×10^7 to 1.3×10^8 for the AOB and 4.1×10^8 to 8.1×10^8 for the N_2-fixing bacteria per g dry soil (Tables 2 and 3). Thus, if gene abundances are used as proxies for the size of the functional communities, the N_2-fixing community was more abundant overall than the ammonia oxidisers, but the ranges of AOA and AOB gene copy numbers between treatments were greater than the range of *nifH* gene copies.

Based on the mean values, all three communities were more abundant in the treatments with brassicas than in the untreated control soil ($P=0.019$) sampled both before and after the pea bioassay in the low N-fertiliser treatments. Community size was also affected by sampling occasion, with the mean abundances being greater on the first soil sampling occasion ($P=0.001$) than on the second occasion, after the pea bioassay. The abundances of the three genes reflecting the different microbial communities were greater in soils with the high N-fertiliser dose than those with the low dose ($P=0.001$) on the second sampling occasion. On the sampling before harvest (low N dose), the number of *amoA* genes from the AOA community was significantly higher ($P=0.017$) in the fertilised control than in the

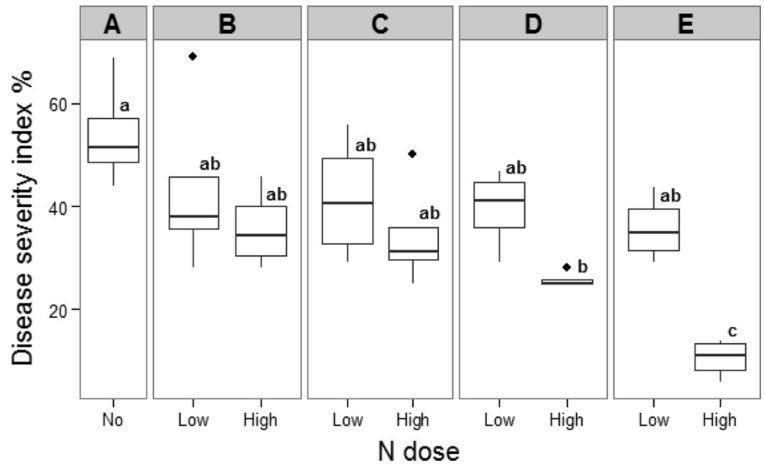

Fig. 1 Incidence of Aphanomyces pea root rot (disease severity index, DSI) on peas grown for 4 weeks in a bioassay after growing cover crops for 11 weeks at a low or high N dose in *Aphanomyces euteiches* infested soil, and removal of both shoots and roots. The treatments with crops were compared to fertilised or unfertilised treatments without cover crop: **a** *unfertilised control*, **b** *fertilised control*, **c** *Secale cereale*, **d** *Brassica juncea* and **e** *Sinapis alba*. The whiskers indicate minimum and maximum values and the points show single outliers. The ANOVA was made on 10-logarithm transformed data and the figure shows the back transformed values. Different letters indicate significant differences between means within each N dose and in comparison with the control ($P<0.05$, Tukey's test, n=4)

S. cereale treatment (Table 2). On the second sampling occasion, the number of *nifH* genes in the low N-dose regime was significantly higher ($P=0.030$) in the *S. cereale* and *B. juncea* treatments than in the fertilised control. In soil samples collected after the 4-week pea bioassay from the high N fertiliser treatment, *amoA* genes from AOB were more abundant ($P< 0.001$) in all cover crop treatments and in the fertilised control compared to the untreated control soil.

Table 1 Pea root nodule formation rate based on an initial 11 weeks of growing a cover crop at a low or high N dose followed removal of cover crop and 4 weeks of pea growth. Different number indicate the rate of nodule formation in each treatment (1=low and 5=high)

Treatment	*Nodule formation*	
	Low N dose	High N dose
Unfertilised control[a]	3	3
Fertilised control[b]	3	2
Secale cereale	5	4
Brassica juncea	3	2
Sinapis alba	2	1

[a] Aphanomyces-infested soil without cover crops and no N added, included in the observations of both Low and High N dose

[b] 1M $(NH_4)_2SO_4$ applied to Aphanomyces-infested soil without cover crops

Associations between pea root rot DSI and N-cycling communities

There were relative differences among the N-cycling communities and the development of Aphanomyces pea root rot in the soils after the pea growth assay in the treatments according to the PCA (Fig. 2). The samples receiving a high N dose separated from the low N dose in the first two axes. In PC1, explaining 60.5 % of the total variance, this was mainly driven by the increase of N-cycling genes and the decrease in development of Aphanomyces pea root rot in the high N samples. Along the second axis, which explained 27.2 % of the variance, the *S. alba* treatment with the high N-fertiliser dose DSI was clearly distinguished from the control treatment, and DSI was associated with N_2-fixing *nifH* genes. The increased abundance of bacterial *amoA* genes in the soils with the high N-fertiliser dose was more associated to the treatments with plants, especially *S. cereale* (PC3, 8.6 % of the variance), indicating a positive effect of plants on this community.

Soil available N-NH_4^+ and N-NO_3^-

The concentration of N-NO^-_3 at the first sampling was higher ($P= 0.001$) in fertilised and unfertilised control treatments than in treatments where cover crops were grown. However, the N-concentrations in the control

Table 2 Abundance (copies g^{-1} dry soil) of the *amoA* gene of ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB) and the *nifH* gene of free-living N_2-fixing bacteria before (first) and after (second) a pea growth assay (based on an initial 11 weeks of growing a cover crop at a low or high N dose followed removal of cover crop and 4 weeks of pea growth)

Treatment	*AOA*[4] (10^8)			*AOB*[4] (10^8)			*nifH*[4] (10^8)		
	First	Second		First	Second		First	Second	
	Low	Low	High	Low	Low	High	Low	Low	High
Unfertilised control[1]	0.70[ab]	0.60[a]	0.60[a]	0.57[a]	0.42[a]	0.42 [b]	6.59[a]	5.61[ab]	5.61[a]
Fertilised control[2]	1.04[a]	0.50[a]	0.59[a]	0.71[a]	0.35[a]	0.93[a]	6.93[a]	4.04 [b]	4.84[a]
Secale cereale	0.58 [b]	0.61[a]	0.69[a]	0.43[a]	0.55[a]	1.31[a]	5.55[a]	6.12[a]	6.10[a]
Brassica juncea	0.92[ab]	0.69[a]	0.88[a]	0.75[a]	0.57[a]	1.05[a]	8.04[a]	5.98[a]	6.79[a]
Sinapis alba	0.78[ab]	0.61[a]	0.68[a]	0.82[a]	0.60[a]	1.19[a]	7.21[a]	5.32[ab]	5.53[a]
Standard error[3]	0.097	0.010	0.008	0.018	0.012	0.009	0.101	0.077	0.089
p-value	0.017	0.404	0.064	0.229	0.061	<0.001	0.288	0.030	0.247

Different letters indicate significant differences between means within N dose and in comparison with the control ($P<0.05$, Tukey's test, $n=4$)

[a] Aphanomyces-infested soil without cover crops and no N added, included in the analyses of both Low and High N dose

[b] 1 M $(NH_4)_2SO_4$ applied to Aphanomyces-infested soil without cover crops

[c] Standard error of transformed means

[d] The ANOVA was made on 10-logarithm transformed data and the figure shows the back transformed values

treatments declined during the bioassay with pea growth, and no significant difference remained after the bioassay. The concentration of N-NH_4^+ in the soil was higher ($P=0.005$) in treatments with cover crops than in the controls on both sampling occasions, and was particularly high in the *S. cereale* treatment.

Table 3 Effects of 11 weeks of cover crop growth at a low N dose on the soil N-NH_4^+ and N-NO_3^- content (mg kg^{-1} dry soil) based on soil samples collected before (first) and after (second) a 4 week pea growth bioassay at a low N dose

Treatment	*N-NH_4^+*[4]		*N-NO_3^-*[4]	
	First	Second	First	Second
Unfertilised control[1]	2.60[c]	2.91[c]	9.04[b]	1.26[a]
Fertilised control[2]	2.63[bc]	3.14[bc]	69.79[a]	2.25[a]
Secale cereale	4.86[a]	4.25[a]	0.62[c]	1.38[a]
Brassica juncea	3.50[abc]	3.58[b]	0.97[c]	2.08[a]
Sinapis alba	4.22[ab]	3.44[b]	1.18[c]	2.51[a]
Standard error[3]	0.21	0.07	0.54	0.53
p-value	0.005	0.001	0.001	0.500

Different letters indicate significant differences ($P<0.05$, Tukey's test, $n=4$)

[a] Aphanomyces- infested soil without cover crops and added N

[b] 1 M $(NH_4)_2SO_4$ applied to Aphanomyces-infested soil without cover crops

[c] Standard error of transformed means

[d] The ANOVA was made on 10-logarithm transformed data and the figure shows the back transformed values

ITCs in the soil-root environment

The quantity of ITC volatiles in the root-soil environment of growing *B. juncea* plants was higher than with *S. alba* (Table 4). The dominant ITC in the *B. juncea* soil was aromatic 2-phenylethyl, which was detected in amounts more than ten times higher than in the *S. alba* soil. Nevertheless, the quantity of aliphatic ITCs was higher and the composition more diverse in the *S. alba* soil than with *B. juncea*.

Discussion

In this study, where the two Brassicaceae species *B. juncea* and *S. alba* were grown in *A. euteiches* infested soil, ITCs were present in the root-soil environment of both brassicas after 11 weeks of growth, but it was primarily *S. alba* that showed inhibitory effects on root rot severity in subsequently cultivated pea plants. There was an interaction with N fertilisation, but the fertilised control treatment without cover crop did not significantly affect the disease severity index, in neither the low nor the

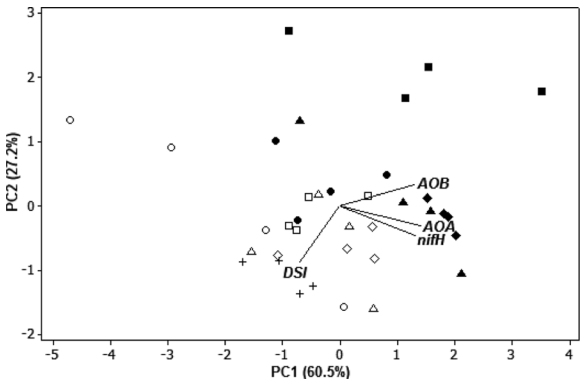

Fig. 2 Principal component (PC) analysis showing the associations between abundances of *amoA* and *nifH* genes from ammonia-oxidising and free-living N_2-fixing microbial communities, and the development of Aphanomyces pea root (disease severity index). The microbial parameters were determined after the pea growth assay, which followed an initial 11 weeks of growing a cover crop at a high (filled) or low (unfilled) N dose. Treatments: *Brassica juncea* (*diamonds*), *Sinapis alba* (*squares*), *Secale cereale* (*triangles*) fertilised control (*circles*) and unfertilised control (*plus*). Abbreviations: DSI, disease severity index; AOA, ammonia-oxidising archaea; AOB, ammonia-oxidising bacteria; *nifH*, gene for nitrogenase in N_2-fixing bacteria. The total variance explained is 87.5 % and the proportion for each PC is indicated

high N dose regimes. Thus, the results support the idea that living roots of certain brassicas can be used to supress *A. euteiches* pea root rot by releasing either ITCs directly or other water soluble products that are rapidly hydrolysed from GSLs in the root-soil environment. It is known that the suppressive effect depends on the ITC composition and concentration (Angus et al. 1994; Matthiessen and Shackleton 2005) and on the exposure time of the organism (Hossain et al. 2014). Our results suggest that only the type and amount of ITCs originating from *S. alba* intact roots were important for reducing the development of pea root rot by suppressing *A. euteiches*. Several studies have shown that aliphatic ITCs exhibit a stronger toxic effect than the aromatic ITCs (Sarwar et al. 1998; Smolinska et al. 2003; Matthiessen and Shackleton 2005; Hossain et al. 2014). Nevertheless, the aromatic 2-phenylethyl ITC detected in the *B. juncea* treatment is known to have toxic effects on several organisms, including mycelium growth of *A. euteiches* as shown by in-vitro tests using synthetic 2 phenylethyl dissolved in a fungal growth medium (Smith and Kirkegaard 2002). If the *A. euteiches* oospores are less sensitive than the mycelium of this pathogen, this could explain why the higher concentrations of 2-phenylethyl in the root-zone of *B. juncea* compared to *S. alba,* did not affect the development of pea root rot in the present study. Matthiessen and Shackleton (2005) showed that the biological activity of ITCs is significantly affected by the presence of soil. They observed that although the aromatic ITCs can exert a strong effect, their activity declines rapidly compared to the activity of aliphatic ITCs. This could explain why the relatively high concentration of aromatic compared to aliphatic ITCs in the *B. juncea* root rhizosphere did not strongly affect *A. euteiches* in our study. Since aliphatic ITCs exhibit a stronger toxic effect than the aromatic ITCs, and were present in higher concentrations and with greater diversity in *S. alba* than in *B. juncea*, this could explain the suppressive effect registered for the *S. alba* treatment. Further, a non-volatile ionic thiocyanate (SCN^-) is normally produced from the sinalbin GSL known to be present in *S. alba* roots, or from the unstable sinalbin hydrolysed ITC 4-hydroxybenzyl (Gmelin and Virtanen 1960; Kawakishi and Muramatsu 1966). Since SCN^- is toxic to various microorganisms (Brown and Morra 1997), this may also have affected the pathogen. The greater suppression of the disease observed at the high N dose could be explained by enhanced cover crop growth rather than a direct N-effect as there was no impact of higher N in control soil. Accordingly, increased N fertilisation would result in a larger root volume and potentially more GSLs being produced.

Disease suppression by the brassicas could also be explained by indirect effects, and multiple mechanisms of action, other than activity of GSL hydrolysis products, have been suggested. Since Brassicaceae amendments have been shown to alter soil microbial communities several authors have proposed possible disease suppressive effects (Omirou et al. 2011; Wang et al. 2014; Mowlick et al. 2013). As in the present study, Rumberger and Marshner (2004) showed ITC release from living roots of canola and suggested that root pathogens may be directly or indirectly negatively affected by ITCs via observed changes in rhizosphere microbial community composition. Mazzola et al. (2012) speculated that the ITCs from rapeseed meal stimulated soil fungi, which resulted in a significant reduction in disease development caused by the plant pathogenic oomycete *Pythium*. However, Cohen et al. (2005) recorded high concentrations of nitric oxide (NO) after rapeseed meal amendments, which could indicate a role for bacterial-derived NO in disease suppression. In the present study we cannot exclude changes in the microbial community in any of the treatments, but potential indirect effects caused by altered

Table 4 Volatile compounds released from growing roots of *Brassica juncea* (cv. Corron) and *Sinapis alba* (cv. Architect) collected in soil during 24 h

Category	ITC	*Brassica juncea*		*Sinapis alba*	
		μg 24h[a]	SE[c]	μg 24h[a]	SE[c]
Aliphatic	Allyl[a]	15.00	5.20	nd[d]	
	Heptyl[a]	nd		7.36	3.07
	3-Butenyl[b]	nd		21.10	9.13
	3-Methylhexyl[b]	nd		9.96	5.79
	4-Methylpentyl[a]	nd		2.39	0.42
Total aliphatic		15.00		40.78	
Aromatic	Benzyl[a]	nd		27.30	14.70
	2-Phenylethyl[a]	486.80	162.10	30.50	13.80
Total aromatic		486.80		57.80	
Other volatile	Dimethyl Trisulphide[a]	43.70	29.50	nd	
Total volatile		545.50		98.60	

[a]mass spectrum and KI match with NIST and authentic standard

[b]mass spectrum match in NIST and KI concurs with KI, published by Valette et al. 2006

[c]SE (Standard Error)

[d]nd (not detected)

communities were only manifested in terms of decrease disease severity in the soil with *S. alba.*

Formation of root nodules was most effective when peas were grown after *Secale cereale* which coincided with the lowest level of NO_3^- after 11 weeks of *S. cereale* growth in the soil. According to Brockwell et al. (1989), a high level of soil N diminishes both rhizobium colonisation and nodulation in soybeans. Thus, the low level of NO_3^- observed in our study likely influenced the rhizobium bacteria to form more nodules in the pea roots. The formation of pea root nodules was lower after Brassicaceae cover crops, especially *Sinapis alba*. The higher amounts of aliphatic ITCs generated by *S. alba* than *Brassica juncea* might have suppressed the activity of the rhizobia. In agreement, Muehlchen et al. (1990) showed that incorporated Brassicaceae tissues in soil significantly reduced the formation of pea root nodules and they suggested that hydrolysed products of GSLs reduce the activity of rhizobium bacteria.

Contrary to our prediction, the brassicas did not have any suppressive effect on the abundance of the N_2-fixing and ammonia-oxidising microbial communities. Wang et al. (2014) showed a direct lowering and persistent lower abundance of ammonia-oxidising bacteria in rapeseed meal treatments, whereas Cohen et al. (2005) reported an increased abundance. Our results suggest that the genetic potential of the N-cycling organisms is not strongly affected by biofumigation efforts using brassicas, which is in line with Omirou et al. (2011) who did not find any effect on the ammonia-oxidising bacterial community in biofumigation treatments with broccoli residues. Other studies have shown that root nodule formation and growth of rhizobia communities were affected by soil incorporated Brassicaceae tissues and living roots of brassicas (Muehlchen et al. 1990; Bressan et al. 2009). Moreover, synthetic ITCs as well as GSLs hydrolysed products from incorporated Brassicaceae tissues can inhibit nitrification (Bending and Lincoln 2000; Brown and Morra 2009). This may be influenced by soil types. Brown and Morra (2009) used a silt-loam and Bending and Lincoln (2000) a sandy- and a clay-loam both with markedly lower clay content than in the present study. The clay soil might have a better buffering capacity. The lack of a negative effect in the current study may be due to differing effects of the various types of ITCs brassicas can produce. Therefore we conclude that the genetic potential of N_2-fixing and ammonia-oxidising communities were less sensitive to the isothiocyanates originating from GSLs in the root-soil environment of the studied Brassicaceae cover crops than was the *A. euteiches* oospores' ability to cause root rot in subsequent peas. Although we cannot exclude the possibility that N_2-fixation and ammonia-oxidation activities were affected by the GSL hydrolysis products, the effect was not strong enough to affect growth to such an extent that it decreased the genetic potential for these important soil functions. Instead, a positive response of the bacterial ammonia oxidisers was seen in all treatments compared to the control in the soils receiving high N-fertilisation. This suggests that the time was sufficient to allow detectable changes in the microbial communities at the

DNA level and that growth occurred. The clear separation of *S. alba* from the control treatment along the second axis of the PCA (Fig. 2) can largely be explained by the low DSI in peas grown after *S. alba* (high N dose), but also by N-cycling communities being relatively more associated to *S. alba* than the control. This indicates that the cover crops have multiple effects on the soil microbial community and that the effect of the cover crops on the subsequent crop is complex.

High concentrations of NO_3^- were detected in the fertilised control without cover crop plants prior to the pea growth bioassay (Table 3). This indicates that ammonia oxidisers in the soil were active and oxidised the ammonium fertiliser to nitrate during the experiment. After the bioassay, the NO_3^- -concentration in the N-fertilised control was reduced to similar levels as in the other treatments. The pea plants probably consumed some NO_3^- and some could have been denitrified and lost, but most of the NO_3^- probably leached, since the peas were irrigated daily with some surplus to enhance disease development, equalize water supply among treatments and avoid drought. Changes in the relative abundances of the bacterial *amoA* genes were associated with N-fertilisation, which confirms previous findings that N-fertilisers affect the nitrifying community (Zhang et al. 2010; Wessén et al. 2011; Levičnik-Höfferle et al. 2012). The significant increase in AOB but not AOA in the high N dose treatments suggests that the AOB reacted more strongly to high N availability than their archaeal counterparts. Ammonia-oxidising bacteria often dominate in nutrient-rich environments (Wells et al. 2009; Di et al. 2010), and it has been suggested that low-nutrient conditions create a potential niche for AOA (Erguder et al. 2009). This is supported by the very low K_m-values for ammonia recorded for the marine archaeon *Nirosopumilis maritimus* and an archaeal soil enrichment when compared with a range of AOB species (Prosser and Nicol 2012 and references therein). Nevertheless, considering the high diversity of terrestrial AOA, there could be organisms with lower affinities. Niche differentiation in relation to N is not fully understood and tolerance of high ammonia concentrations and the source of ammonia as well as other soil factors may also be important, since no single soil factor seem to discriminate between AOA and AOB (Prosser and Nicol 2012). Interestingly, the size of the AOA community was smallest in the *S. cereale* treatment when sampled before harvesting the cover crop. The *S. cereale* crop could have been more competitive when it comes to N-uptake since it produced a larger root volume than the Brassicaceae cover crops, according to observations at the time of cover crop harvest. Another explanation could be that the growing roots of *S. cereale* inhibited the AOA community by releasing root exudates (Kruidhof et al. 2008). As reviewed by Subbarao et al. (2012), forage grass species and species within cereal crops like Sorghum have shown biological nitrification inhibition.

The relative increases in the abundances of functional genes from the microbial N-cycling communities were negatively correlated with the development of pea root rot, and were associated with the high N dose regime. Thus, the overall positive effect of N addition on the N-cycling community could explain part of the decrease in DSI. Soil microbial biomass and microbial activity are generally positively correlated with the suppression of soil borne diseases such as *Pythium* damping-off of maize and bean root rots (Darby 2003). Matthiessen and Kirkegaard (2006) also suggested that organic N in soil promotes the growth of soil organisms and indirectly affects soil-borne pathogens. Nevertheless, the abundance of *nifH* genes was high in the unfertilised control treatment showing the most severe development of pea root rot. Since N_2-fixing rhizobium bacteria (Maxwell et al. 1989) and zoospores of *Aphanomyces* spp. (Tahara et al. 2001) are both attracted by the gradient of flavonoids released from pea roots, the incidence of Aphanomyces pea root rot and the abundance of the N_2-fixing community may be positively correlated. It has been suggested that the mineral salts have a direct suppressive effect on Aphanomyces by increasing the osmotic pressure in *A. euteiches* cells and reducing their activity (Lewis 1973). Another suggested mechanism by which Aphanomyces infection can be reduced by nutrients is that the nutrients make the pea root tissues more woody in character and difficult for the pathogen to infect (Smith and Walker 1941). In addition, N can have many effects on the soil microbial community that are not investigated here, but that could affect Aphanomyces infection.

We conclude that 11 weeks growth of Brassicaceae cover crops until flowering did not decrease the genetic potential of the soil N_2-fixing and ammonia-oxidising communities, despite the fact that the brassicas did suppress Aphanomyces pea root rot. This suggests that growth of Brassicaceae cover crops does not negatively influence the presence of key N-cycling organisms in the soil. However, further in-depth studies may reveal

negative long-term effects on the activity of these organisms, or on other soil organisms with important ecosystem functions.

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