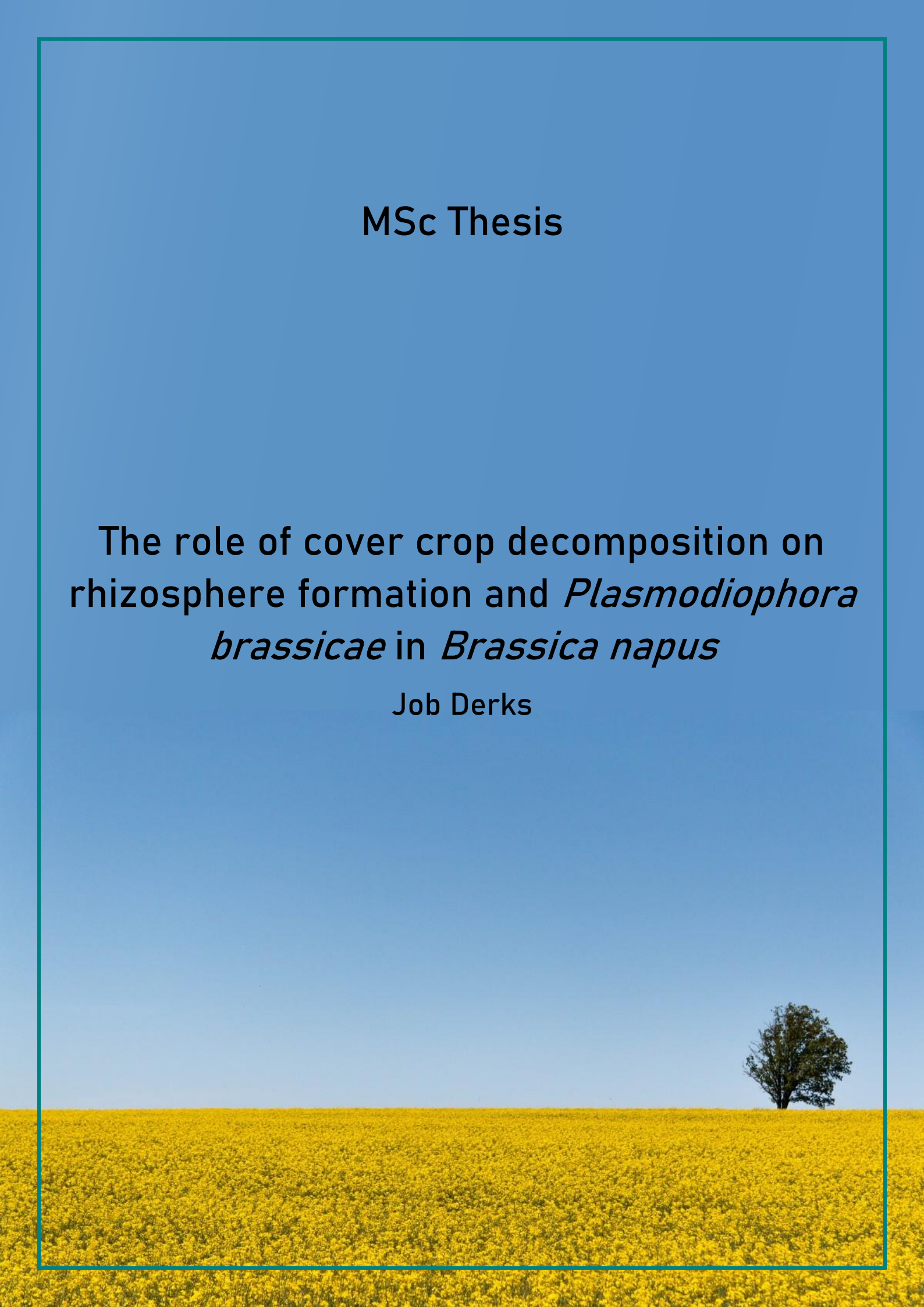
MSc Thesis

The role of cover crop decomposition on rhizosphere formation and *Plasmodiophora brassicae* in *Brassica napus*

Job Derks



MSc Thesis

The role of cover crop decomposition on rhizosphere formation and *Plasmodiophora brassicae* in *Brassica napus*

Job Derks

Registration number: 1021012

Wageningen University, Laboratory of Nematology

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MSc Plant Sciences – Plant Pathology and Entomology

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Abstract

Clubroot, caused by *Plasmodiophora brassicae* is a persistent soilborne disease threatening global oilseed rape production. Infection of a suitable brassica host leads to the formation of galls and subsequent release of resting spores which can remain detectable for up to 17 years. Methods to control this soilborne pathogen are limited by restrictions and bound to environmental and health concerns. However, steering the oilseed rape rhizosphere through specific cover crop incorporation may alleviate disease pressure. Quantity and quality of plant residue greatly differ between plant species offering opportunities to affect soil properties like pH and soil microbiome composition differentially. In a greenhouse experiment, we decomposed eight cover crops (black oat, camelina, Egyptian clover, Ethiopian mustard, flax, niger seed, oilseed radish and vetch) after which clubroot spores were added and oilseed rape was grown. Subsequently, overall fungal, bacterial, nematode and clubroot gene copy numbers were compared after decomposition and after plant growth to see if clubroot abundance was affected. Differences in fungal to bacterial ratios after decomposition were found in black oat, niger seed, oilseed radish and Ethiopian mustard treatments, and F:B ratio could be correlated to pH. No differences in abundance or ratio were found for the organisms analysed in the rhizosphere, but the oilseed radish treatment led to significantly better plant growth than other treatments. Underlying mechanisms in which cover crop quality influences fungal and bacterial abundance and ratios remain to be determined. Ultimately, unravelling the steering effect plant residue can have on the soil microbiome may offer durable clubroot control methods in the future.

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List of abbreviations

- Bla: black oat
- C:N ratio: carbon to nitrogen ratio
- Cam: camelina
- Con: Control
- Egy: Egyptian clover
- Eth: Ethiopian mustard
- F:B ratio: fungal to bacterial ratio
- Fla: flax
- Nig: niger seed
- Rad: oilseed radish
- Vet: Vetch

Introduction

The *Brassicaceae* (*Cruciferae*) is an important plant family consisting of 360 genera and 4000 species found on every continent except Antarctica (Jabeen, 2020). Brassica species are applied as model plants in research (*Arabidopsis thaliana*), used as ornamentals in gardens, and are important fodder, medicinal, vegetable and oilseed crops (Raza et al., 2020). Worldwide, 36.5 million hectares are cultivated with brassicas of which oilseed rape (*B. napus)* alone makes up 34 million ha of the total acreage of brassicas (FAOSTAT 2019). Canada is a big producer of oilseed rape with 8.6 million ha (Statistics Canada, 2019), but also in Europe, oilseed rape makes up 54% of the total oilseed crop production (European Commission, 2019). However, intensive cultivation has caused the accumulation and spread of soilborne diseases, such as clubroot (Gossen et al., 2015).

Clubroot, caused by the protist *Plasmodiophora brassicae*, leads to an average brassica yield loss of 10 to 15% globally, but 100% crop loss can occur locally (Dixon, 2009a). A major issue is the survival structure of the disease: extremely persistent resting spores. If the soil is minorly infested, planting a single generation of susceptible plants can already increase the spore load by over 60-fold (Hwang et al., 2013). Moreover, these resting spores may remain detectable for up to 17.3 years (Wallenhammar, 1996). Resting spores germinate in the presence of root exudates of clubroot hosts as well as some non-host plants (Macfarlane, 1970; Rashid et al., 2013). From every resting spore, a primary zoospore is released that can initiate root hair infection. Zoosporangial clusters develop in the root hairs and release secondary zoospores that infect cortical cells. The cortex infection induces hyperplasia and hypertrophy of the root cells which results in the characteristic clubroot galls (Kageyama & Asano, 2009). These galls disrupt water- and nutrient uptake, which results in stunted growth, wilting and sometimes death of the plant (Strelkov et al., 2006). After disintegration of the gall, millions of new resting spores get released into the soil (Hwang et al., 2012).

Effective disease control is challenging because of the persistent nature of resting spores. Preferably, the disease is prevented before it gets established in the host. Current agrochemical control methods are limited by restrictions and bound to environmental and health concerns. Other control methods include the use of resistant cultivars, long crop rotations and calcium and boron amendment (Donald & Porter, 2009). Nevertheless, the continuous use of resistant cultivars favours a rapid proliferation of more virulent clubroot pathotypes that can break resistance (Cao et al., 2020; Strelkov et al., 2018). Besides, even though a crop rotation of more than 2 years results in a 90% reduction of resting spores, the remaining spores still affect yield in non-resistant cultivars (Peng et al., 2015). Alternatively, management techniques that involve the use of soil microbial life might be an effective control strategy to inhibit resting spore germination or to prevent root hair infection (Bhering et al., 2020).

Microorganisms can contribute to plant-health by activating defence mechanisms in the plant, directly antagonising pathogens, or by competing with the pathogen for resources (Raaijmakers et al., 2009). The most enriched microbial communities that interact with plants are found in the rhizosphere (i.e. the soil directly surrounding the plant root), while more specific microbiomes are found in the endosphere (all inner root tissues) (Kuzyakov & Razavi, 2019; Vandenkoornhuyse et al., 2015). The composition of these communities in early seedlings can largely be predicted by what microbes initially inhabit the soil (Walsh et al., 2021). These microbes are legacies that plants leave in the soil via rhizodeposits and litter, and are retained in the roots of young plants (Hannula et al., 2021). Decaying plant material and associated microbes (the detritusphere) are considered a microbial hotspot and affect the rhizosphere more than the plant type itself (Zhou et al., 2020), but the information is conflicting (Hannula et al., 2021). Microbes seem promising in bio pest control as they are key in regulating plant pests such as *Ralstonia solanacearum*, *Verticilium dahlia* and *Rhizoctonia solani* (Kwak et al., 2018; Lazcano et al., 2021; H. Liu et al., 2021), as well as clubroot (Lebreton et al., 2019; Saraiva

et al., 2020). By steering root microbiomes, disease tolerance may be enhanced (Han et al., 2021; X. Liu et al., 2021; Yuan et al., 2021).

Incorporating cover crops into the soil is an attractive method to steer soil microbiomes (X. Liu et al., 2021). Cover crops are planted to i.e. prevent soil erosion, improve water holding capacity and soil fertility or suppress weeds (Lu et al., 2000). Based on the cover crop quantity and quality, residues can strongly impact growth through nutrient cycling or variable suppression or stimulation of diseases (Bonanomi et al., 2010; Qi et al., 2020). However, the quality and quantity of cover crops greatly differ among plant species and also differentially steer soil microbiomes (Fanin et al., 2014; X. Liu et al., 2021). Quality depends on the ratio of carbon to nutrients and easily decomposable C (labile) forms versus more complex (recalcitrant) forms like lignin (Cotrufo et al., 2013). High carbon to nitrogen (C:N) ratios and a greater proportion of recalcitrant C stimulate fungal- over bacterial abundancy in soils and vice versa (Bossuyt et al., 2001; Cotrufo et al., 2013; Grosso et al., 2016). Besides, adding cover crop residue may increase pH because of the decomposition of organic anions and the ammonification of residue N (Vanzolini et al., 2017; Xu & Coventry, 2003). Higher pH levels positively correspond with microbial biomass but lower the fungal to bacterial ratio (Bååth & Anderson, 2003). For clubroot, crop rotation and adding rice straw influenced the fungal and bacterial abundance and led to the suppression of clubroot (Han et al., 2021; Zhang et al., 2022). Such variation in detritusphere as imposed by different crops may thus offer novel clubroot control strategies.

Still literature of specific crop effects on clubroot suppression remains sparse. Therefore, this research aims to elucidate the impact of the detritusphere- and rhizosphere microbe interactions in enhancing or suppressing clubroot in oilseed rape. Eight different cover crops were decomposed after which *P. brassicae* resting spores were added and a susceptible oilseed rape cultivar was grown. We predict that i) differences in cover crop type affect oilseed rape growth, ii) cover crop type influences the fungal and bacterial abundance and ratio in the detritusphere, (iii) differences in the fungal and bacterial abundance and ratio between treatments is also reflected in the rhizosphere composition of oilseed rape, and (iv) differences in fungal and bacterial abundance and ratio affect clubroot incidence.

Materials & Methods

Plant material and soil preparation

Cover crop material for decomposition was collected on November 25th, 2021 from a field plot of Joordens Zaden Company, situated near the village of Kessel, the Netherlands (51.2774°N, 6.0161°E). Eight cover crops were included: black oat (*Avena strigosa*, Bla), camelina (*Camelina sativa*, Cam), Egyptian clover (*Trifolium alexandrinum*, Egy), Ethiopian mustard (*Brassica carinata*, Eth), flax (*Linum usitatissimum*, Fla), niger seed (*Guizotia abyssinica*, Nig), oilseed radish (*Raphanus sativus*, Rad) and vetch (*Vicia sativa*, Vet). Collected crop material, consisting of shoots and roots, was rinsed with water to remove excess soil, centrifuged in a vegetable centrifuge, dried in a stove at 70°C for 3 days and chopped into fragments of ~5cm.

An arable soil for organic brassica cultivation was collected at Unifarm, Wageningen (51.9906°N, 5.6654°E) to make a microbial inoculum. From the arable soil, a soil subsample was taken, sieved with a 2 mm mesh sieve and frozen in liquid nitrogen to be stored at -20°C until DNA extraction. A soil suspension was prepared by mixing 21 kg of the arable soil with 15.75 L of demineralised water as described in van de Voorde et al. (2012). Additionally, sieved potting soil was mixed with river sand (ratio 50:50). From this mixed soil, pH was measured in a 1:2.5 soil/demineralised water suspension with a pH electrode. Water holding capacity was determined using 40 g of soil in containers with small drainage holes as described in Haney and Haney (2010).

Resting spore isolation

Clubroot galls were washed until clean and homogenised in tap water in a blender. The suspension was filtered through one layer of cheesecloth, one layer of cheesecloth in a 500 µm mesh sieve, and then through a 100 and 45 µm mesh sieve. The sieved suspension was centrifuged at 2000 rpm for 10 min (Beckman CP centrifuge) and the supernatant was discarded. Resting spores were washed three times with sterilised demineralised water. The resulting resting spores were surface sterilised with ethanol and washed an additional two times with sterilised demineralised water by centrifugation at 14.000 rpm for 30 sec (Eppendorf Centrifuge 5417R). To determine the concentration of spores, spores were resuspended in 1 mL sterilised demineralised water and counted with a counting chamber (Bürker-Türk Bright-Line). Resting spore pellets were stored at -20°C until use (see 'Plant Decomposition & Resting Spore Inoculation' and 'Bioassay for testing Detritusphere Volatile - Resting Spore Interactions').

Cover crop decomposition & resting spore inoculation

For every cover crop species, 210 g of dry plant material was mixed with 10 kg of soil and put in bags. Next, the bags were autoclaved twice for 4.5 h at 120°C, 2.5 bar to obtain completely sterile soil. Circular plastic pots (2 L, upper diameter 16 cm, lower diameter 12.5 cm, and height 13 cm) were filled with 1 kg of plant-soil mixture per pot after which 195 mL arable soil suspension was inoculated. As a control, ten pots did not include plant material. Pots were labelled per decomposing plant species, put in a controlled greenhouse environment and arranged in a randomised complete block design, with ten replications per treatment which made a total of 90 labelled pots. The pots were watered with demineralised water to 65% water holding capacity and were left to decompose for 9 weeks (Figure 1). Pots were watered twice a week with irrigation water up to 65% water holding capacity. Mean day and night temperatures were 21°C and 16°C respectively. The light regime consisted of a 16h photoperiod and the relative humidity was around 70%.

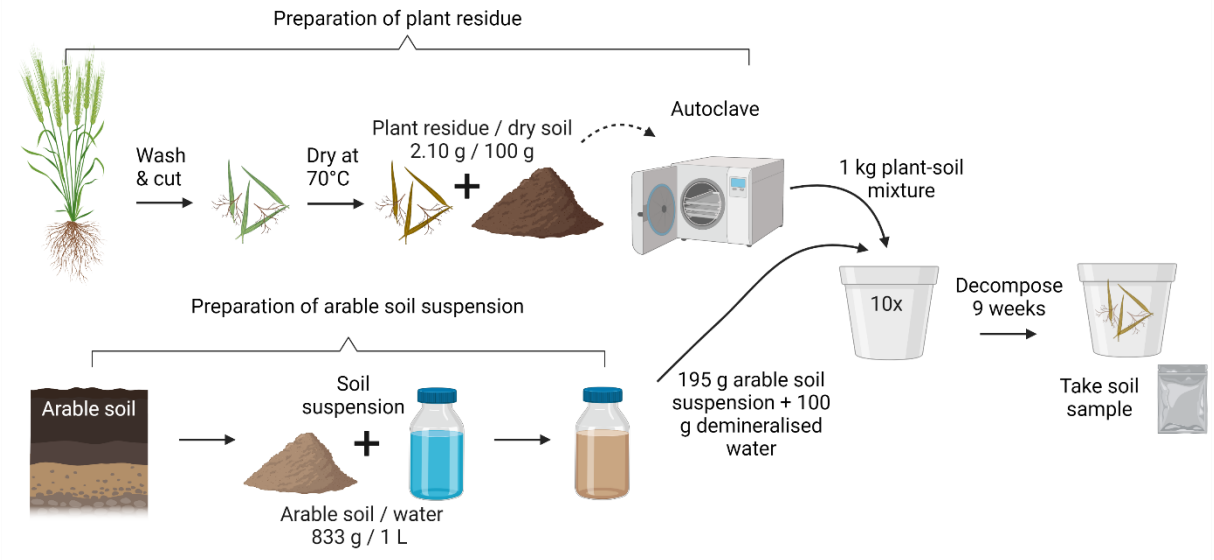

Figure 1. Workflow before decomposition. Plant material was harvested, washed, dried and mixed with prepared soil. The plant-soil mixture was autoclaved twice to obtain completely sterile soil. For the arable soil suspension, organic arable soil was sieved and mixed with demineralised water. Per plant-soil mixture, 10 pots were filled and put randomly in a greenhouse to decompose for 9 weeks after which a soil sample is taken. Created with BioRender.com.

After incubation, every replicate was handled separately. The soil was sieved with a 2 mm mesh sieve to filter out residue. Samples were taken and frozen immediately in liquid nitrogen to be stored at 20°C until DNA extraction. An additional soil sample of each treatment was made and stored at 4°C (see

'Bioassay for testing Detritusphere Volatile - Resting Spore Interactions'). To correct any abiotic changes that may have occurred during decomposition, two times 10% of incubated soil was mixed with 90% of novel sterilised mixed soil prepared according to the same procedure. Pots were maintained under the same conditions as for decomposition. Six days later, *P. brassicae* spore suspension was mixed in to achieve an inoculum density of 7.4 x 10^4 resting spores per g of soil.

Rapeseed growth

Five spring oilseed rape seeds of variety Helga were sown per pot and allowed to grow for one week, after which they were thinned until one plant remained. These plants were grown for six weeks, after which disease indices were determined, pH measured and soil- and root samples taken (Figure 2) (see 'DNA extraction and quantitative PCR'). Rapeseed shoots were dried and weight was measured.

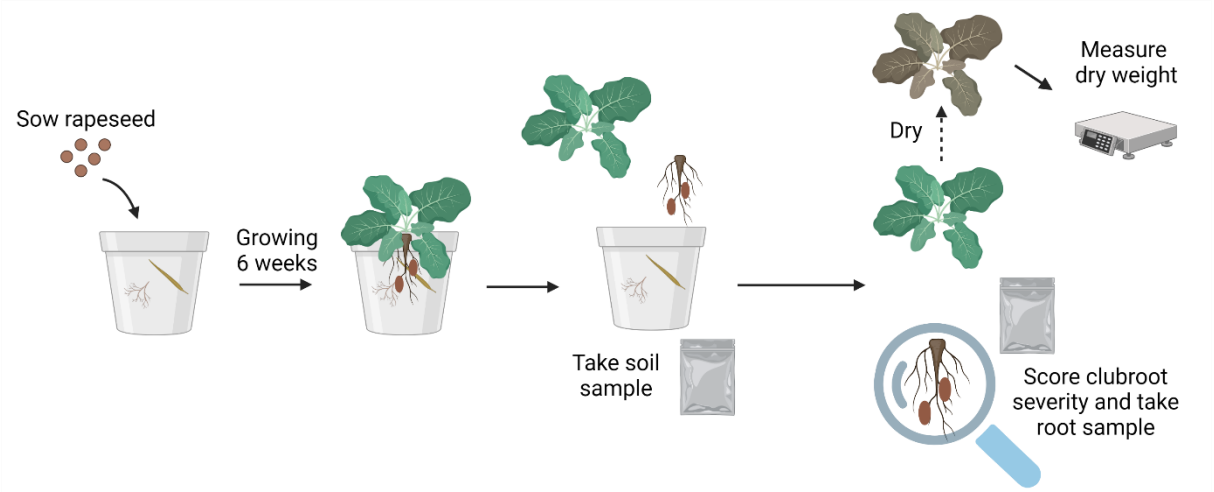

Figure 2. Workflow for rapeseed plant growth and subsequent plant-growth and disease assessment. Five rapeseed seeds were sown per pot, and after one week, plants were thinned until one plant remained. These plants were allowed to grow for another six weeks after which plants were removed. Rhizosphere and roots were sampled and the plants were scored for clubroot severity. Also, dry weight was measured. Created with BioRender.com.

DNA extraction and quantitative PCR

After plant growth, samples were taken from the rapeseed rhizosphere. The roots had colonised the entire pot, and therefore, soil that adheres to the roots after softly shaking the plants was considered rhizosphere. Rhizosphere soil was collected and stored at -20°C until DNA extraction. Fine hair roots were removed until the primary root with lateral roots remained. Subsequently, soil particles adhering to the roots were rinsed with tap water after which the roots were checked for clubroot.

DNA was extracted from the detritusphere soil and rhizosphere soils according to a lab-made protocol (Table S1). This protocol uses 1 g of soil weighed in a 15 mL tube as a starting point. First, the soil is physically disrupted in bead solution using silicon carbide powder and a vortex (Vortex Genie 2 Digital). Then, the samples are centrifuged for 2 minutes at 2.500 g (Hereaus Multifuge 3 S-R) to separate soil particles from the lysate. Non-DNA, inorganic matter and humic acids were precipitated and nucleic acids were re-dissolved in a binding solution. DNA was further purified using AcroPrep Advance Filter 1 mL Plates for DNA Purification (PALL). The quantity of the obtained DNA was measured with a Qubit DNA BR Assay kit (ThermoFisher). Quantitative PCR was used for the quantification of total bacterial, fungal and nematode communities and clubroot. Amplification of the bacterial 16S ribosomal DNA (rDNA) was performed using primers 341F and 534R. Amplification of the fungal ITS1-5.8S rDNA with primers ITS1F and 5.8S and nematode 18S small subunit rDNA with primers 1912 R and 1480 F. qPCR

results were corrected for soil water content and primer efficiencies. For clubroot, a proxy primer efficiency was taken. Details for the qPCR procedure are described in Table S2

Bioassay volatile - resting spore interactions

To test the effect of detritusphere-associated volatiles on clubroot resting spore viability and germination, a bioassay was performed in two-compartment Petri dishes (ø 9 cm), as modified from Li et al. (2020). One compartment was prepared with 4 g of sieved soil that was collected after decomposition in the greenhouse, and the other with resting spore suspension (2 x 10^7 spores per mL). As a control, the same was done with sterilised soil. In total, there were n = 45 units: eight greenhouse treatments of which the first five replicates were used, plus five times freshly sterilised soil. All Petri dishes were sealed with 2 layers of Parafilm to minimise gas exchange and incubated in a box at 20 °C for 3 days.

Evans blue was applied to distinguish viable from non-viable resting spores (Harding et al., 2019). In addition, empty spores were assessed to distinguish germinated from non-germinated resting spores. From every treatment, 500 µl of spore suspension was taken and concentrated 25 times by centrifugation. 10 µl spore suspension was mixed with an equal volume of Evans blue stain solution (20 mg/mL) and left for approximately 24 hours at room temperature. After decomposition, each sample was placed on a slide and examined under a light microscope (Imager A2, Zeiss) at 40x magnification. Pictures were taken, and the number of unstained spores (viable), stained spores (non-viable) and empty spores (germinated) was counted with the ImageJ plugin Cell Counter (Figure 3).

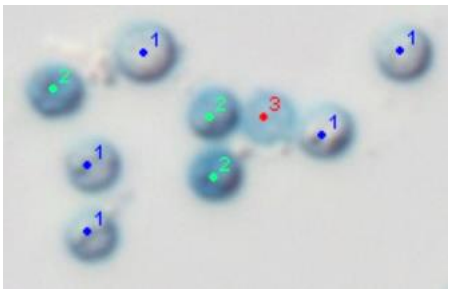

Figure 3. Clubroot resting spores (magnification 40x) stained with Evans blue. 1 = viable spores, 2 = dead spores 3 = germinated spores.

Statistical analyses

All data gathered in this experiment was statistically analysed in R version 4.1.2 with R Studio (R Core Team 2021). Fungal, bacterial, nematode and clubroot gene copy numbers were corrected for dilutions made during DNA extraction and qPCR analysis. Differences in pH, plant growth and fungal, bacterial, nematode and clubroot abundances between treatments were assessed by an ANOVA or Kruskal-Wallis test depending on normality and homogeneity of variances. The clubroot gene copy number found in the soil was divided by the gene copy number of fungi, bacteria and nematodes to check for differences in population density. Also, ratios fungi to bacteria (F:B), fungi to nematodes (F:N) and bacteria to nematodes (B:N) were calculated and differences were assessed between treatments by an ANOVA or Kruskal-Wallis test. The F:B ratio was compared to the pH and to the amount of clubroot using a Spearman's rank correlation test.

Data that followed a normal distribution and showed homogeneity of variances was analysed using ANOVA followed by a Tukey-HSD post hoc test. In case of nonnormality or inequality of variances, a Kruskal-Wallis test was performed followed by a Dunn post hoc test using the Holm adjustment method. To increase statistical power, one outlier for vetch after decomposition was removed in the bacterial qPCR and one vetch outlier in the F:B ratio to clubroot correlation. Additionally, one outlier in the control was removed for the comparison in the ratio of clubroot to fungi, bacteria and nematodes.

Results

pH measurements

After decomposition, the mean pH between treatments ranged from 6.35 to 6.65 and differences were present between treatments (Kruskal-Wallis, chi-squared = 20.156, $P < 0.01$) (Table 1). Soils were most basic for niger seed and most acidic for oilseed radish ($P < 0.05$). After plant growth, pH values were more equal than after decomposition, ranging from 6.03 to 6.16, but differences between treatments were still found (ANOVA, $F = 2.915$, $P < 0.05$) (Table 1). With a difference in pH of 0.13, oilseed radish had a higher pH than the control ($P < 0.05$). Also the change in pH between decomposition and after growth of oilseed rape was different (ANOVA, $F = 11.15$, $P < 0.001$) (Table 1). Significant differences were present between in the control and black oat ($P < 0.05$), control and oilseed radish ($P < 0.05$), oilseed radish and camelina, Ethiopian mustard, flax, vetch ($P < 0.01$) and oilseed radish and black oat, Egyptian clover, niger seed ($P < 0.001$).

Table 1. Soil and crop characteristics per treatment. Average pH ± SE after cover crop decomposition, after oilseed rape growth, the change in pH between decomposition and plant growth ± SE of oilseed rape in gram. Letters on superscript indicate pairwise differences ($P < 0.05$) using Dunn's test for multiple comparisons for pH decomposition and pH growth and Tukey's honestly significant difference test for Δ pH change.

Treatment	**pH decomposition**	**pH growth**	**Δ pH change**
Control	6.65±0.026^{ab}	6.03±0.032^{b}	0.52±0.052^{b}
Black oat	6.81±0.086^{ab}	6.11±0.022^{ab}	0.75±0.088^{a}
Camelina	6.64±0.026^{ab}	6.12±0.009^{ab}	0.53±0.039^{ab}
Egyptian clover	6.74±0.028^{ab}	6.09±0.017^{ab}	0.71±0.037^{ab}
Ethiopian mustard	6.71±0.027^{ab}	6.06±0.026^{ab}	0.54±0.038^{ab}
Flax	6.67±0.030^{ab}	6.1±0.027^{ab}	0.56±0.022^{ab}
Niger seed	6.82±0.025^{a}	6.08±0.020^{ab}	0.73±0.042^{ab}
Oilseed radish	6.35±0.019^{b}	6.16±0.014^{a}	0.25±0.025^{c}
Vetch	6.63±0.018^{ab}	6.13±0.029^{ab}	0.54±0.034^{ab}

Plant dry weight

Variation in plant growth was observed at the end of the experiment (Figure 4), and differences in growth were also observed in the dry weight (DW) (Kruskal-Wallis, chi-squared = 48.039, $P < 0.001$). Oilseed radish treated soils led to plants that were 2.2 times higher in DW than the control and 7 times higher than black oat treated soils. Significant differences in DW were present between black oat and Ethiopian mustard, oilseed radish (1.65 and 3.97 g, respectively, $P < 0.001$), Ethiopian mustard and flax (1.28 g, $P < 0.05$), oilseed radish and Egyptian clover, flax (3.54 and 3.60 g, respectively, $P < 0.001$) and oilseed radish and niger seed (3.34 g, $P < 0.01$) (Table 1).

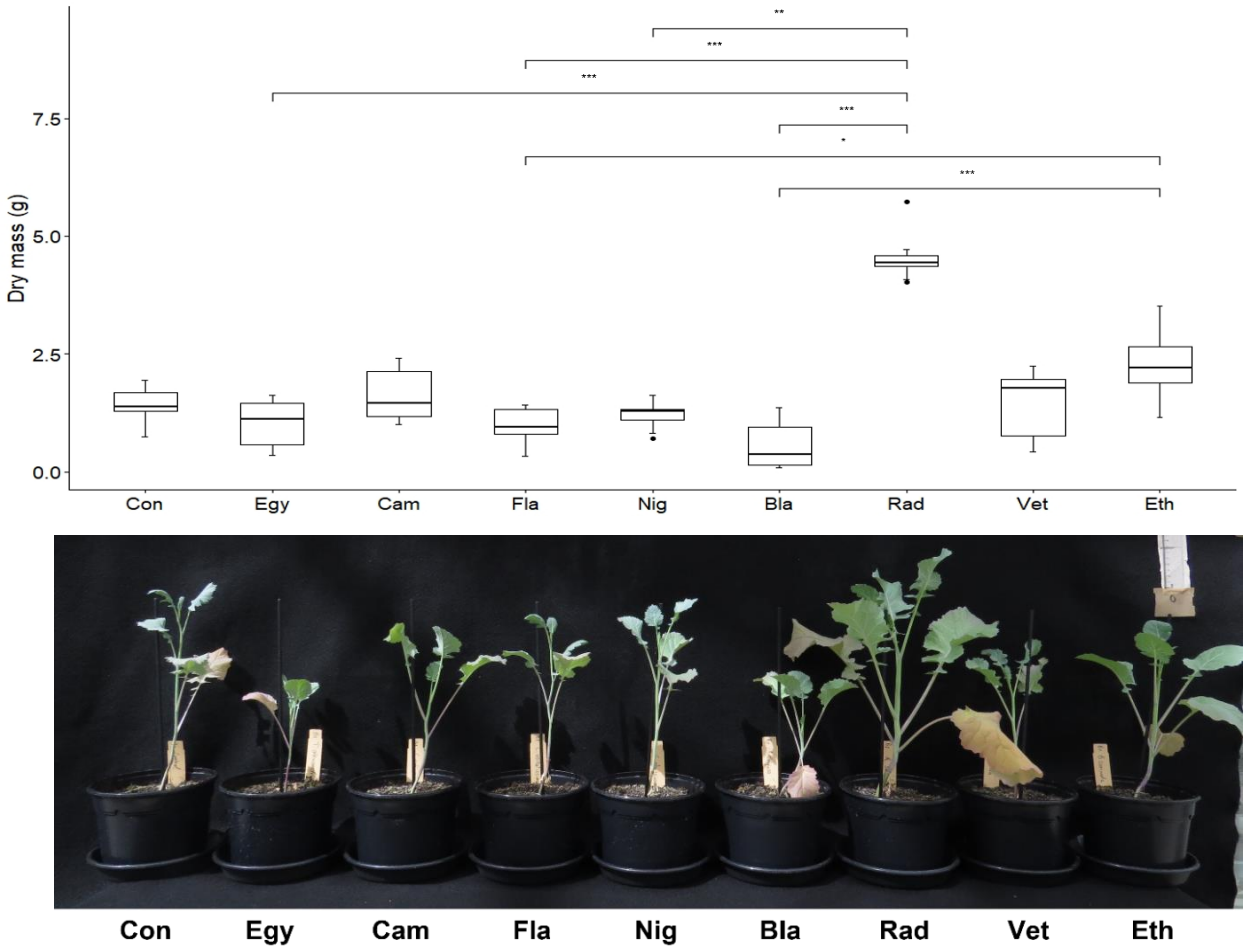

Figure 4. Difference in mean dry mass per treatment (g) and visualisation of a representative block. A Kruskal-Wallis test was conducted to check for differences between treatments after decomposition ($P < 0.001$). A Dunn's test for multiple comparisons using the Holm method was performed to compare individual treatments. Only significant differences are shown. *P*-values < 0.001 are indicated with '***', values < 0.01 with '**', and values < 0.05 with '*'.

Clubroot incidence

None of the treatments showed any clubroot symptoms.

Fungal, bacterial and nematode gene copy numbers

No significant differences were found when comparing the fungal (Kruskal-Wallis, chi-squared = 5.9061, $P = 0.6578$), bacterial (Kruskal-Wallis, chi-squared = 2.5285, $P = 0.9604$) and nematode (Kruskal-Wallis, chi-squared = 4.3246, $P = 0.8267$) gene copy numbers after decomposition (Figure S6). After growth, the copy numbers of all groups had dropped and values became more equal between treatments for fungi and nematodes. None of these differences was significant in fungal (Kruskal-Wallis, chi-squared = 6.1612, $P = 0.561$), bacterial (ANOVA, $F = 1.194$, $P = 0.33$) and nematode (Kruskal-Wallis, chi-squared = 10.437, $P = 0.2357$) gene copy numbers. Also no significant differences were found when comparing the ratio fungi to nematodes after decomposition (Kruskal-Wallis, chi-squared = 8.5785, $P = 0.3791$), and bacteria to nematodes after decomposition (Kruskal-Wallis, chi-squared = 5.9976, $P = 0.6474$) and plant growth (Kruskal-Wallis, chi-squared = 12.046, $P = 0.1491$). In contrast, differences were present in the fungal to nematode ratio after plant growth (Kruskal-Wallis, chi-squared = 16.543, $P = 0.03524$) (Figure S7). However, no significant pairwise difference was found in the post-hoc Dunn test.

Additionally, F:B ratios did differ between treatments after decomposition (Kruskal-Wallis, chi-squared = 25.468, $P < 0.01$). Black oat and niger seed had the lowest average F:B ratios of 0.19 and 0.16. The leafy brassica treatments Ethiopian mustard and oilseed radish were the highest at 0.37 and 0.40. Ratio's F:B differed between oilseed radish and niger seed ($P < 0.01$), oilseed radish and black oat ($P < 0.05$) and niger seed and Ethiopian mustard ($P < 0.05$). These differences in F:B ratio were negatively correlated with pH (Spearman's rank correlation, *rho* = -0.4745302, $P = 1.1*10^{-3}$) (Figure 5). After plant growth, ratios were more equal between the treatments and no significant differences in ratio were present (ANOVA, $F = 0.927$, $P = 0.506$).

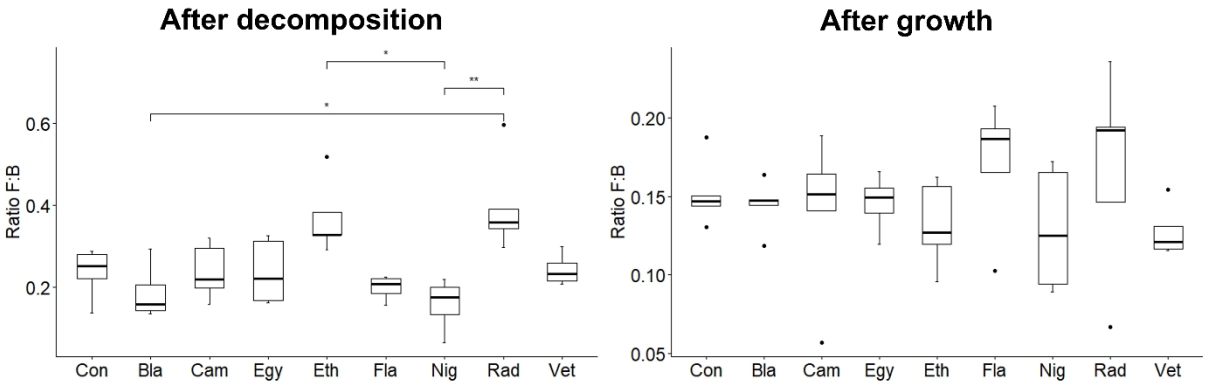

Figure 5. The ratio of fungi to bacteria (F:B) after cover crop decomposition and after oilseed rape growth. A Kruskal-Wallis test was conducted to check for differences between treatments after cover crop decomposition ($P < 0.01$), and an ANOVA for differences between treatments after oilseed rape growth ($P > 0.05$). A Dunn's test for multiple comparisons using the Holm method was performed to compare individual treatments. Only significant differences are shown. *P*-values < 0.01 are indicated with '**', and values < 0.05 with '*'.

Differences in the Δ change F:B between treatments were found (Kruskal-Wallis, chi-squared = 23.265, $P < 0.01$). The change in F:B ratio was most pronounced for Eth, and was significantly different from Fla and Nig ($P < 0.05$) (Figure 6).

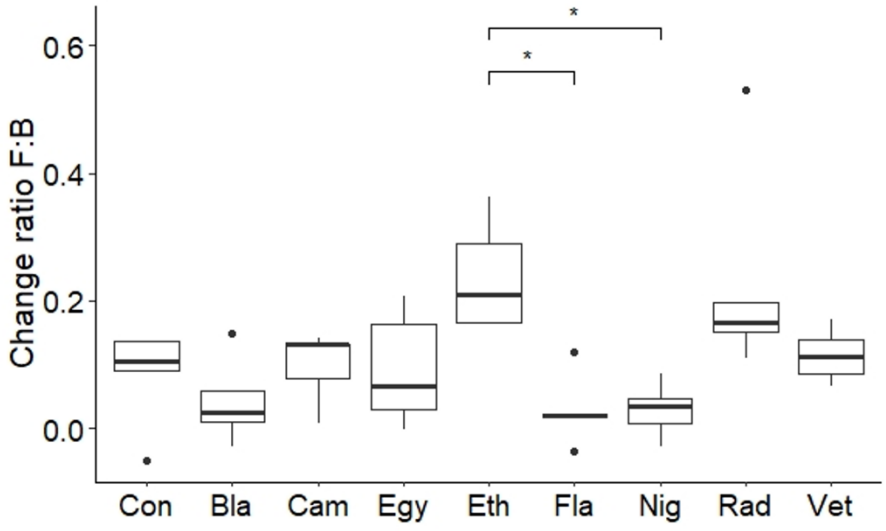

Figure 6. Change in ratio fungi to bacteria (F:B) between cover crop decomposition and oilseed rape growth. A Kruskal-Wallis test was conducted to check for differences between treatments ($P < 0.01$). A Dunn's test for multiple comparisons using the Holm method was performed to compare individual treatments. Only significant differences are shown. *P*-values < 0.05 are indicated with '*'.

Clubroot gene copy number

Analysis of qPCR data showed no significant differences in clubroot gene copy number between treatments (Kruskal-Wallis, chi-squared = 2.1009, *P* = 0.9778) (Figure S8A). No significant differences in ratio were found for fungi to clubroot (Kruskal-Wallis, chi-squared = 9.1624, *P* = 0.3288), bacteria to clubroot (Kruskal-Wallis, chi-squared = 5.2767, *P* = 0.7276) and nematodes to clubroot (Kruskal-Wallis, chi-squared = 9.0485, *P* = 0.3382) (Figure S8B,C,D). Further analysis indicated no relation between the F:B ratio and the amount of clubroot (Spearman's rank correlation, *rho* = 0.216, *P* = 0.106) (Figure S9).

Bioassay volatile – resting spore interactions

No significant differences were present in the amount of viable spores between treatments (ANOVA, *F* = 1.805, *P* = 0.127). Treatments did not vary in the amount of nonviable spores (ANOVA, *F* = 0.127, *P* = 0.127), or germinated spores (ANOVA, *F* = 0.79, *P* = 0.602). Also when comparing the ratio of viable to nonviable spores, no significant differences were found (ANOVA, *F* = 1.816, *P* = 0.125) (Figure S10).

Discussion

Black oat leads to poor plant growth and oilseed radish to lush plant growth

Black oat treated soils led to the development of small plants. It is especially plants in this treatment that started to show red hypocotyls during growth. Red hypocotyls in oilseed rape have previously been reported under mineral nutrient deficiencies and as a result of seedling blight, with a range of possible soilborne pathogens (Bruce et al., 2006; Hwang et al., 2014; Hodges & Nozzolillo, 1996). It is unlikely that nutrient deficiencies played a role as plants were grown in a fresh substrate of sand and potting soil, of which the potting soil should contain enough nutrients for the experiment. More likely is the influence of a pathogen. Particularly soil moisture and temperature play a big role in the development of seedling blight in oilseed rape (Hwang et al., 2014). Although soil water content was the same in every treatment, the black oat treatment seemed very wet and may have been more affected by seedling blight, reducing plant growth. Contrarily, the Ethiopian mustard and especially oilseed radish treatment supported the growth of big plants (Figure 4). Because novel sterilised soil was inoculated before plant growth and any major abiotic effects on plant and pathogen performance were removed, it is assumed that differences in growth are caused by biotic factors. No correlation was found between F:B ratio and DM, but it is expected that differences in plant growth are a result of intraspecific community composition.

Cover crop type induces differences in F:B ratio and pH

In partial support of our hypothesis, differences in the F:B ratio between the niger seed treatment and two of the brassica treatments, Ethiopian mustard and oilseed radish, and the black oat and oilseed radish treatment after decomposition were found (Figure 5). No differences were found in fungal, bacterial or nematode abundance. After decomposition of the eight different cover crops, pH varied significantly between treatments. Oilseed radish treatments yielded more acidic soils whereas niger seed treatments were the most basic (Table 1). At first, decomposition of organic anions and ammonification may have increased soil pH, but in the end nitrification leads to lower pH values (Vanzolini et al., 2017; Xu & Coventry, 2003). Following other studies, our study found lower pH values to be correlated to an increase in F:B ratio (Bååth & Anderson, 2003; Rousk et al., 2010). It is thus possible that the F:B ratio was influenced by the pH.

Differences in detritusphere F:B ratio are not maintained in the rhizosphere

In opposition to our hypothesis, differences in F:B ratio after decomposition were not significant anymore in rhizosphere composition (Figure 5). Also pH values were more equal between treatments after plant growth and only differences between the control and oilseed radish were found (Table 1).

Similar pH values across treatments may have led to similar F:B ratios among treatments or vice versa. An equalising effect can also be attributed to the addition of 90% novel sterilised soil to 10% of the soil after decomposition. Removal of plant litter as a source of physical-chemical variation and similar community richness between inoculums may have caused similar development when colonising novel sterile soil (Delmont et al., 2014). Besides, disturbance of the detritusphere may have impaired its capability to degrade root exudates (Zhou et al., 2020), and enhanced the oilseed rape-specific effect on the rhizosphere composition (Gkarmiri et al., 2017). This plant effect increases over time, and may therefore have overruled the effect of the coloniser community structure (Hannula et al., 2021).

No clubroot infection or differences in gene copy numbers

We could only test the last hypothesis partly because none of the treatments showed symptoms of clubroot after plant growth even though temperature, pH and moisture should have allowed for clubroot development (Dixon, 2009b). Evans blue staining of the spores revealed that many of the spores were dead. Hill et al. (2022) show a spore mortality rate of over 70% after 1 minute of exposure to 70% ethanol. In the current study, spores were exposed to 70% ethanol for longer than 1 minute, probably killing off most of the clubroot spores. Additionally, the clubroot pathotype may not have allowed for oilseed rape infection as an experiment using the same spore stock within our group did cause infection in other plants of the Brassica family, but not in oilseed rape.

No differences were found in the amount of clubroot DNA between treatments (Figure S8A), but there seems to be a non-significant trend between the F:B ratio and clubroot gene copy number (Figure S9). At higher F:B ratios, higher clubroot gene copy numbers seemed detectable. It is known that bulk soil- and rhizosphere communities play a key role in controlling pathogens such as clubroot (Daval et al., 2020; Saraiva et al., 2020). However, few papers were found that report on the correlation between F:B ratio and pathogen suppression (Ge et al., 2021; Shen et al., 2013). More research is needed to see if the F:B ratio truly affects the amount of clubroot.

In the volatile experiment, due to differences in the ratio of viable-nonviable spores between the control and soils treated with cover crops (Figure S10), there may be a reason to assume that microorganisms affect the viability of resting spores. It is known that certain bacteria produce antifungal volatiles to grow at the expense of fungi; in oilseed rape, volatile production by the genus *Bacillus* showed inhibitory effects against the development of *Sclerotinia sclerotiorum* (Ribeiro et al., 2021), and ample evidence of volatile-induced inhibition is available for other plant-pathogen systems (Garbeva & Weisskopf, 2020). In our study, we found no evidence for an effect on the resting spore mortality and germination in our set-up. However, due to technical difficulties of the experiment, we cannot draw any conclusion.

Cover crop quality may have affected microbiome composition

At cover crop harvest, niger shoots were brown and partly decayed whereas oilseed radish had big green leaves. In a study by X. Liu et al. (2021), cover crops that turned brown had lower nutrient contents. Such a decrease in the tough, withered niger in contrast to easily accessible nitrogen in the oilseed radish leaves may have caused rapid residue decomposition and nitrification in the latter one. Nevertheless, fungi generally benefit from a low-quality residue (high C:N ratio) whereas bacteria benefit from a high-quality residue (Bossuyt et al., 2001). In our experiment, the green, leafy brassica treatments Ethiopian mustard and oilseed radish are expected to have provided low C:N residue which should result in a low F:B ratio, and niger seed and black oat high C:N residue, resulting in a high F:B ratio. However, such an effect was not found.

Additionally, Brassica-specific glucosinolates (GSLs) may have exerted their biocide function in Ethiopian mustard and oilseed radish. The major toxic compounds responsible for biocontrol like

isothiocyanates (ITCs) are released upon hydrolysis of GSLs by myrosinase, a degrative enzyme present in plants alongside GSLs, and in microbes (Bhat & Vyas, 2019). Drying and autoclaving degraded the myrosinase in cover crops, but the enzyme could have been produced by microorganisms that reside in the soil (Gimsing et al., 2006). Even though initial disease suppression is dependent on bioactive compounds, long-term suppression is mediated by the microbial community (Mazzola et al., 2015). Therefore, Ethiopian mustard and oilseed radish may have shaped a distinct, ITC-tolerant microbial community (Siebers et al., 2018).

Conclusion

In conclusion, the characterisation of overall fungal, bacterial and nematode communities gave an insight into the detritusphere and rhizosphere assembly as established by a diverse range of cover crops. First of all, the oilseed radish treatment showed remarkable growth of plants. Differences in F:B ratios were found in black oat, niger seed, oilseed radish and Ethiopian mustard, and differences in ratio could be correlated to pH. Such differences in community composition disappeared after plant growth. No conclusions can be drawn on the effect of rhizosphere composition on clubroot infection, as no infection was found. However, when comparing the ratio of clubroot to fungi, bacteria and nematodes and the amount of clubroot to F:B ratio, there tends to be a correlation between clubroot gene copy number and the F:B ratio.

In contrast to previous research, cover crops that are expected to have a low C:N ratio did not lead to lower F:B ratios. Therefore, future research should focus on the relation between the nutritional content of cover crops, and the effect on pH and detritusphere composition. Sequencing of the rhizosphere could elucidate remarkable growth in oilseed radish treated soil relative to other treatments. Besides, spores could be taken along in the decomposition process to decipher the role of its associated microbes on resting spore persistence in the soil. It is also possible that fungal and bacterial legacies are maintained inside of the root (the endosphere) (Hannula et al., 2021). Analysis of root samples should reveal if detritusphere composition affects endosphere composition and clubroot infection rate. Soil-microbiome analysis can then be performed to check for correlation between F:B ratio and the amount of clubroot DNA.

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Supporting information

Supplementary methods

Table S2. Protocol: Microbial DNA isolation from 1 gram of soil

Step	Procedure
1.	Weigh 1 g of thoroughly mixed soil, transfer it to a 15 mL bead tube), and add 1.5gr of silicon carbide powder (grid 46).
2.	Add 3 mL of bead solution **C0** (181 mM disodium phosphate, 121 mM guanidinium thiocyanate), 0.24 mL of lysis buffer **C1** (150 mM NaCl, 4% (w/v) SDS, 0.5 M Tris). *If Solution C1 is precipitated, heat solution to hand warm (max. 60 °C) until the precipitate has dissolved before use.*
3.	Bead beat the tubes for 10 minutes in a paint shaker or a vortex (use standardized settings to compare).
4.	Centrifuge the tubes for 2 minutes (2,500 x g) to separate the soil particles from the lysate.
5.	Transfer 1.5 mL of the upper aqueous phase to a new 15 mL tube, and add 0.75 mL of 1.33M ammonium acetate **C2** and hand mix ~1min. *Optional: add 80µl Internal Control DNA*.
6.	Centrifuge the tubes for 2 minutes (2,500 x g) to separate the precipitate from the nucleic acids.
7.	Transfer 1.95 mL (2 x 980µl) of the upper aqueous phase to a new 15 mL tube, and add 0.6 mL of 120 mM ammonium aluminum sulfate dodecahydrate solution **C3**. Shake 1 minute by hand to mix. *If you want a break you can incubate the samples longer than 5 minutes in 4°C (max 12 hours) there is no significant loss of DNA yields or purity seen*
8.	Centrifuge the tubes for 2 minutes (2,500 x g) to separate the precipitate from the nucleic acids.
9.	Avoiding the pellet, transfer a fixed volume (e.g. 1.5 mL) of supernatant into a clean 1.5-15 mL tube (depending on volume).
10.	Add 2 volumes binding solution **C4** (at RT) to the supernatant (binding solution: an aqueous solution of 5M guanidinium thiocyanate and 30mM Tris-HCl (pH: 6.5) with 9% (v/v) isopropanol), and shake by hand to mix.
11.	Place a pall plate (PALL8032) on a vacuum manifold, turn on the vacuum system and load your sample (2 x 3 x 600 µl, so 3.6ml in total) on the pall plate (Figure S1).
12.	Before the wash step remove waste and perform 1 extra spin step for 1 minute (2500 x g) to dry the pellet.
13.	Add 1 mL of washing solution **C5** to the pall plate or spin filter column (10mM Tris-HCl - pH: 6.5, 100mM NaCl, and absolute EtOH final v/v 50%). Do this 2x in total (2 mL).
14.	Before the final elution step remove waste and perform 1 extra spin step for 1 minute (2500 x g) to dry the pellet.
15.	Air-dry the filter for 5 minutes, put a collection plate underneath the vacuum manifold and add 0.2 mL of elution buffer **C6** (10mM Tris-HCl, pH: 8.0) on the column or pall plate filter without touching it. Centrifuge again for 1 minute (2000 x g) The liquid now contains the DNA.
16.	Collect the eluate (0.2 mL) and store it at -20 °C until further use (Figure S2).

Solution	Content	Comments ±250rxn's
C0	181mM Na_2HPO_4 (disodium phosphate Mw:177.9; or pipet from 1M stock B49)----------24.14g 121mM GITC (Guanidinium thiocyanate $C_2H_6N_4S$ Mw:118.16 g/mol C44)----------10.7g *After autoclaving cover bottle with aluminium foil to keep it from light.* Solution C0 is a solution that disperse soil particles, dissolve humic acids and protect nucleic acids from degradation	750ml
C1	0.5M Tris (pH **Not adjusted** Mw:121.14 F14)----------4.85g 4% SDS W/V (Mw:288.3 A30)----------3.2g 150mM NaCl (Mw:58.44g/mol E15)----------0.7g Autoclave Solution C1 contains SDS, an anionic detergent that breaks down the cell membrane	80ml
C2	1.33M $C_2H_7NO_2$ (Ammonium acetate; Mw:77.08g/mol C66) ----------30.75g Autoclave Solution C2 precipitates non-DNA organic and inorganic matter that can inhibit the PCR reaction	300ml
C3	120 mM $NH_4Al(SO_4)2*12H2O$ (aluminum ammonium sulfate dodecahydrate; Mw:453.33 A2)----------16.3g Note: Add $NH_4(Al)SO_4$ after autoclaving MQ ±20min 121°C to prevent precipitation! Solution C3 is another inhibition (humic acids) removing solution	300ml
C4 Option 1	5M Guanidinium thiocyanate (GUSCN or GITC) (C44 Mw118,16)----------443.1g 30mM Tris-HCl pH8.0 F14----------22.5ml 1M stock Add after autoclaving: 9% Isopropanol----------67.8ml *Autoclave GuHCL+Tris-HCl in Endvolume-9%. After autoclaving add the additional isopropanol and cover bottle with aluminium foil to keep it from light.* In Gu Isothyocianate both ions are chaotropic, whereas in the HCl salt only guanidine is, so GuITC is a more powerful denaturant. It's also less soluble Solution C4 is a high concentration salt solution. DNA binds tightly to silica filters at high salt concentrations, which will be the next step of protocol	750ml 682.5ml excl isoprop
C5	100mM NaCl; Mw58.44 g/mol E15----------2.9g 10mM Tris-HCl (**Adjusted** to pH 8.0 F14) ----------5ml 1M stock 50% Ethanol (Add EtOH after autoclaving) *After autoclaving add an equal volume of 99.9% ethanol.* Solution C5 is an ethanol based wash solution that removes residual salt, humic acid and other contaminants while leaving the DNA bound to the filter	500ml

Vortex equipment:
Digital Vortex genie 2 Model: SI-A256 max 2850rpm.

# 15ml Tubes in adaptor	Set speed (rpm)	Effective speed* (rpm)	Position tubes	comments	How to place tubes (lids on inside)
4	2850	**~2540**		Only option ~2540 is optimal for DNA/RNA extraction	

*Effective RPM when max speed setting is used

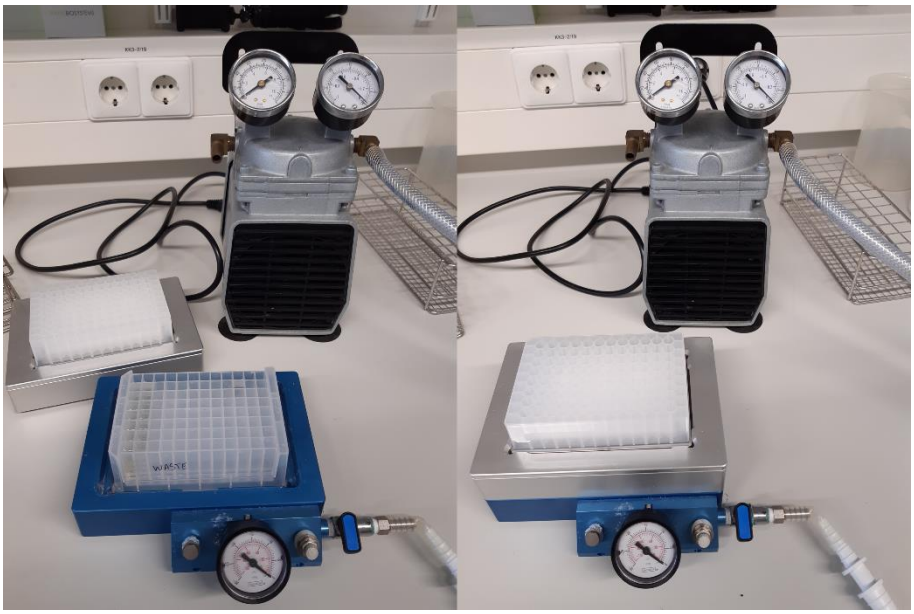

Figure S1. First, a waste plate is placed on the vacuum manifold (left), after which the PALL plate is placed on top (right).

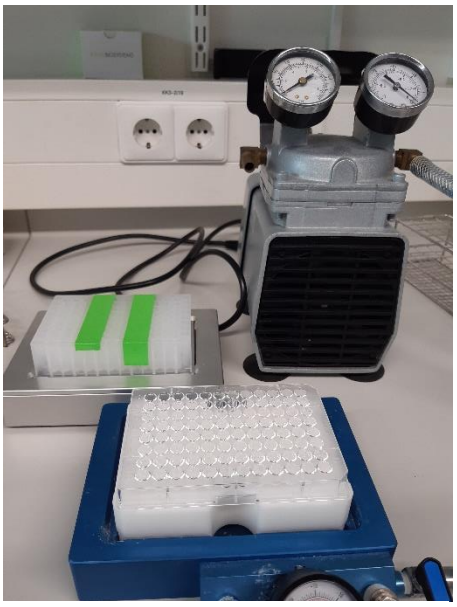

Figure S2. A cell culture plate is put on a white block to reach the correct height, after which eluate is collected.

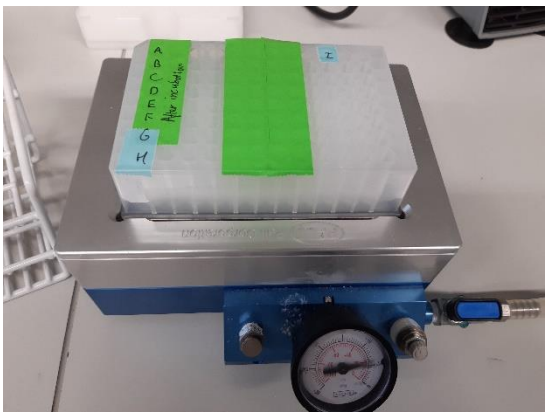

Figure S3. Some cells are taped off to increase suction.

Table S3. Protocol: preparation of qPCR reaction mix

1. Clean the table
2. Make a master mix (in orange) per sample of interest as follows
    a. First add the IQ mix, then the Mili-q, then primers.
    b. Make 10% extra
    c. Vortex or invert carefully to prevent bubble formation

q-PCR reaction mix	
1 µl	Forward primer (5 µM)
1 µl	Reverse primer (5 µM)
5 µl	MQ (always new tube)
10 µl	IQ mix
3 µl	Template
20 µl	TOTAL

1. Per well, add 17 µl of the Master Mix (in green)
2. Per well, add 3 µl of your (diluted) template. Add it on the side of the well and **don't** mix it by pipetting
3. As a negative add 3 µl of mili-q water in 2 wells
4. Close the qPCR plate with a film
5. Spin the plate with a counterweight

Materials & Methods - Check Evans Blue as a viability stain

Because it seemed to vary how many spores were stained per time and sample, we hypothesised that there is a change in viability depending on the time spores were out of the freezer. To this end, two samples of resting spore suspension (4.62 x 10^9 spores per ml) were prepared and a subsample was checked for viability with Evans Blue staining as described before. The spore suspension was stored in the fridge for two days after which viability was checked again.

Results - Check Evans Blue as a viability stain

Because Evans blue staining assumed high mortality of freshly thawed spores, it was tested if spores need time after thawing to become active before excluding Evans Blue from their systems. Staining directly after thawing led to 43% of spores being stained and thus considered dead. Staining one and two days after thawing led to 70% and 76% of spores being stained respectively (Figure S4).

Discussion - Check Evans Blue as a viability stain

Thawing spores and keeping them out of the freezer for a couple of days did not increase activity. On the contrary, an increase in spores stained blue indicates a decrease in activity (Figure S4). Recently, a 2-year experiment found that soil samples stored at 4°C and 20°C better maintained spore viability than at -20°C (Zahr et al., 2021). In contrast, it is common practice to store clubroot galls at -20°C for up to several years (Cruz et al., 2009; Dixon, 1976). Differences in spore viability could be attributed to water content. It has been proposed that preserving spores in dry soil or galls may minimise cell crystallisation, which may damage the cell (Zahr et al., 2021). Because it is unknown what exactly causes mortality in pure spore samples after thawing, more research is needed.

Supplementary figures

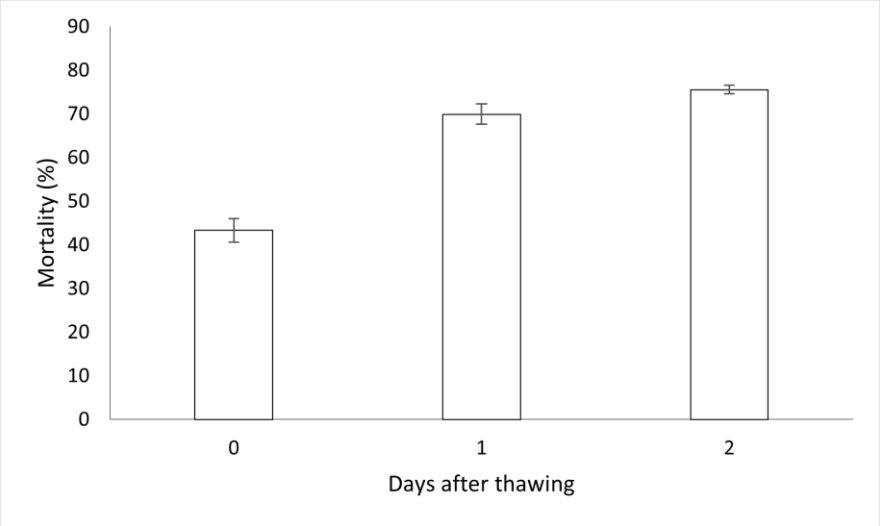

Figure S4. Percentage of stained resting spores with Evans Blue with increasing days after thawing. Stained spores are considered dead. Data shown are averages ± SE.

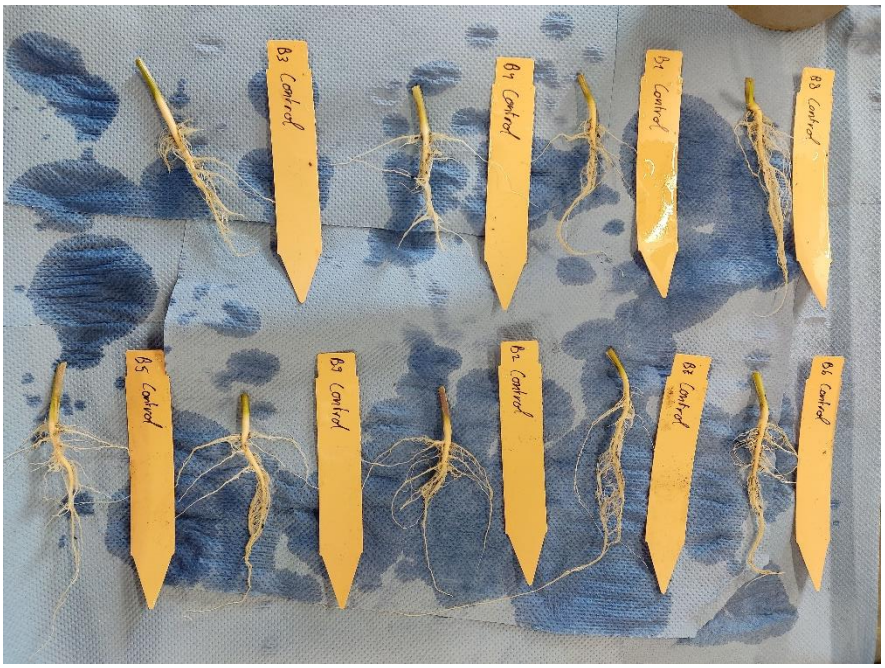

Figure S5. Oilseed rape roots of the control after harvest. No clubroot symptoms are visible.

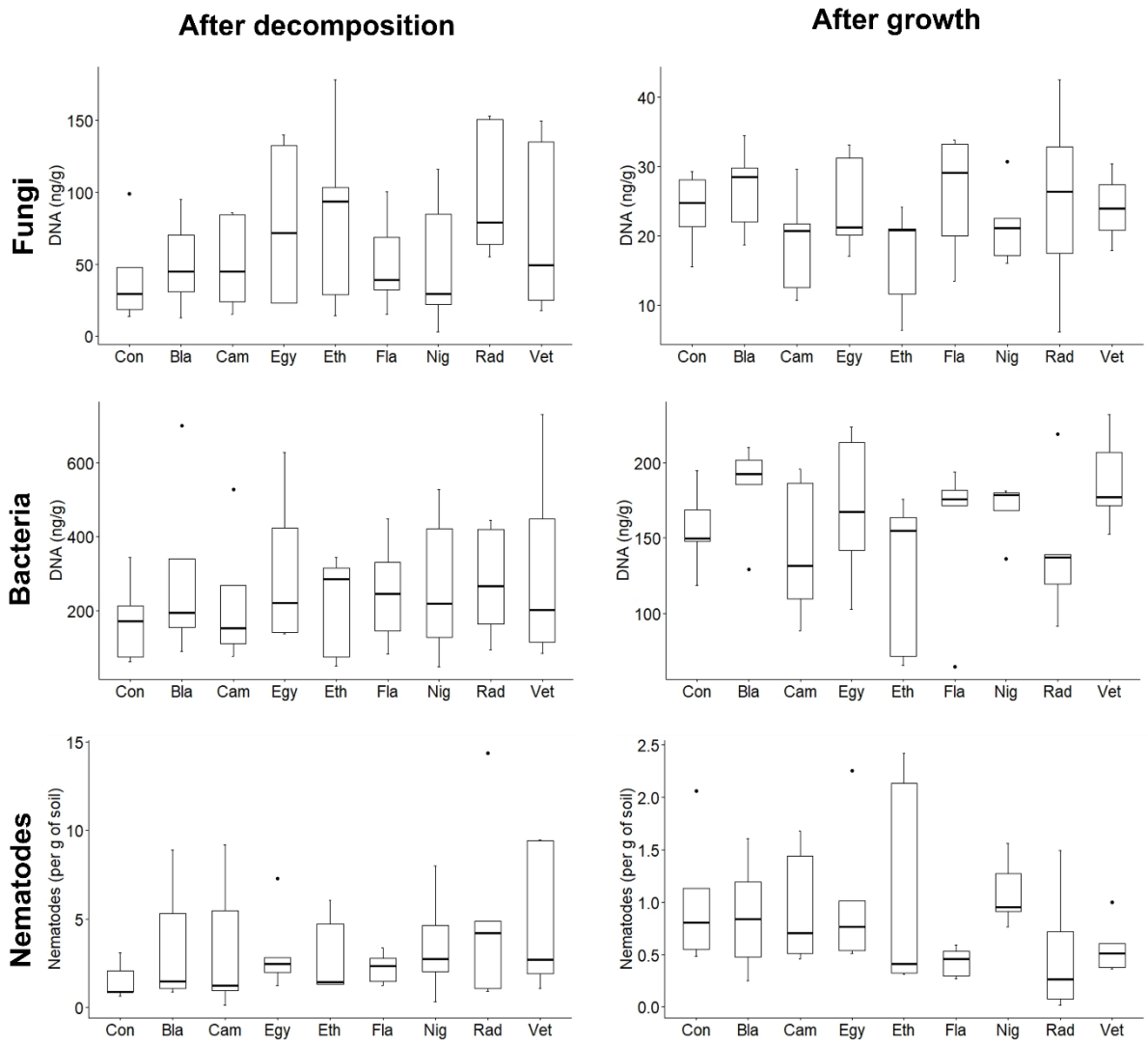

Figure S6. Analysis of fungal, bacterial and nematode gene copy numbers after decomposition of cover crops and after oilseed rape growth. Amount of DNA in ng/g for bacteria and fungi and the number of nematodes in g/soil. No significant differences were found between treatments.

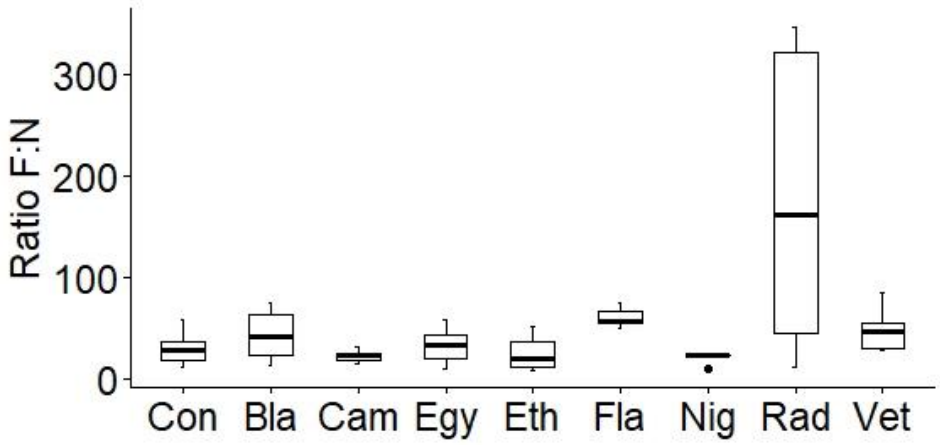

Figure S7. The ratio fungi (F) to nematodes (N) after plant growth.

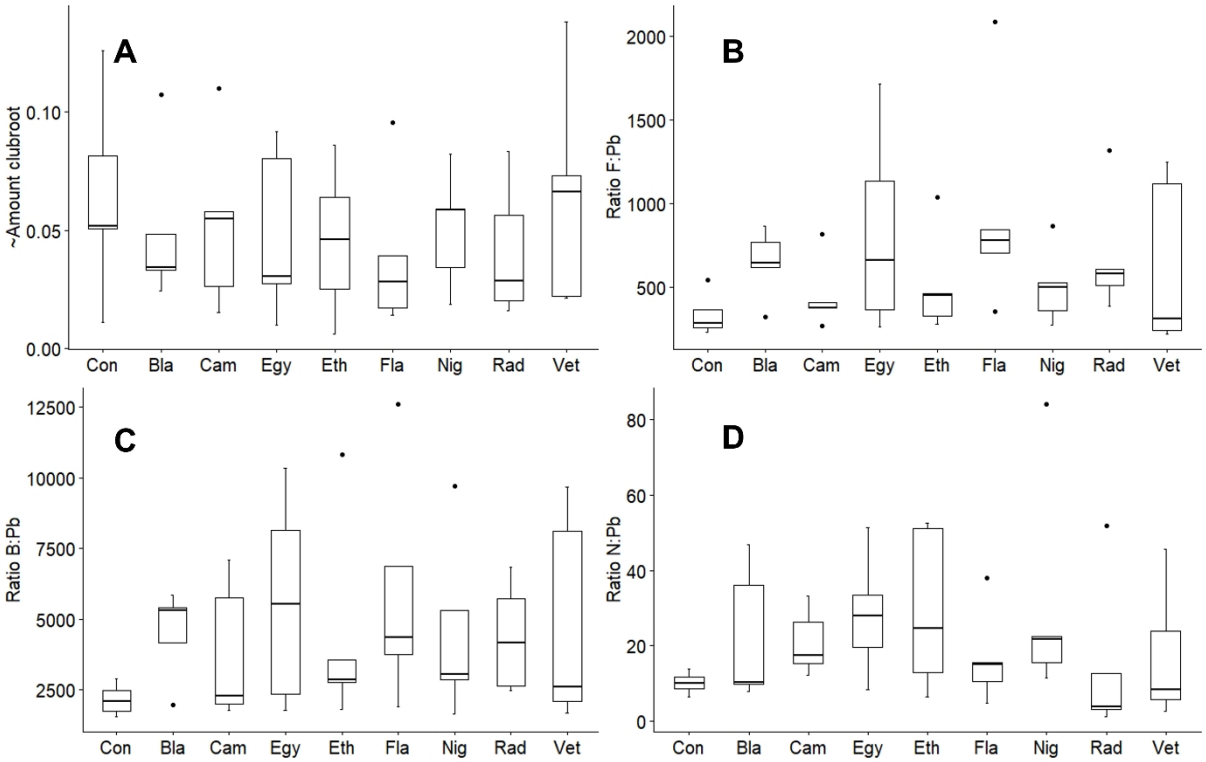

Figure S8. Relative abundance of clubroot in one gram of soil and the ratio of clubroot to fungi, bacteria and nematodes. A: The amount of clubroot in one gram of soil. B: The ratio of fungi (F) to clubroot (Pb). C: The ratio of bacteria (B) to clubroot (Pb). D: The ratio of nematodes (N) to clubroot (Pb).

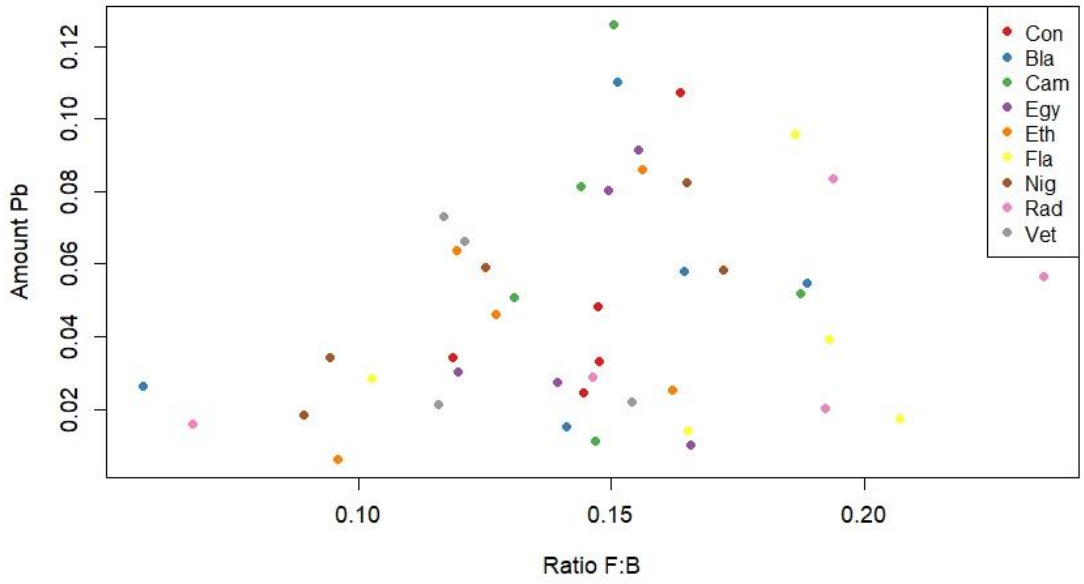

Figure S9. Correlation between the amount of clubroot (Pb) and the ratio fungi to bacteria (F:B). A Spearman's rank correlation test was conducted to check if there is an association between the amount of clubroot and the ratio fungi to bacteria (P = 0.1061).

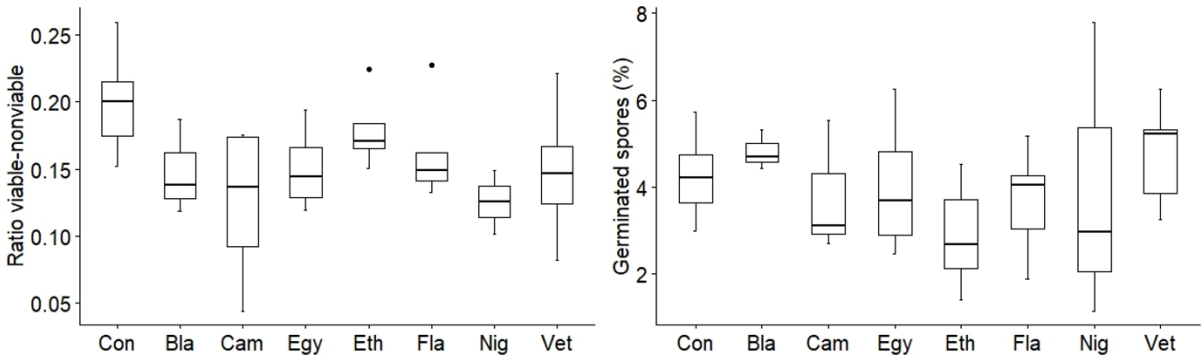

Figure S10. Ratio of viable to nonviable clubroot resting spores and germination of resting spores after exposure to detritusphere volatiles. Based on Evans Blue staining. Left: ratio viable to nonviable resting spores. Right: percentage of germinated resting spores.