The effect of selected cover crops on the antagonists of *Pratylenchus penetrans* and *Rhizoctonia solani*.

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1. Preface

In light of completion of my master thesis there are a lot of people who deserve special thanks for helping me. First of all, I would like to thank Sara Cazzaniga for her contribution in every step of this research. Among other reasons, her patience with regards to my limited laboratory skills were key in completing this thesis. Secondly, I would like to thank Hans Helder for his contributions over the whole course of this thesis as well. I would like to thank Sven Van den Elzen for his insights related to the primer design within this thesis. Moreover, I would like to thank Joeke Postma, Mirjam Schilder and Johnny Visser for supplying me with the pathogens for inoculations and the means to inoculate them. Then I would like to thank Stefan Geisen for letting me use his greenhouse compartment and Carin Lombaers for helping me with the *Rhizoctonia solani* qPCR. Lastly, I would like to thank my examiners, Aska Goverse and Hans Helder, for their upcoming examination.

Since the list of people who helped me is far too big to name everyone individually, I would like to thank the staff and students of the Nematology chair group and Unifarm, both at Wageningen University and Research. Over the course of the research there always has been somebody around to whom I could discuss any problems or uncertainties that arose. Moreover, I would like to thanks the Nematology chair group for facilitating the requirements for this research, especially during the uncertainties related to the COVID pandemic.

2. Abstract

Since the agricultural sector is urged to adopt more environmentally friendly procedures, it is clear that the current conventional way of combatting soil-borne pests and diseases should change as well. A promising method to mitigate soil-borne pathogens in a sustainable manner is the inclusion of antagonists, microorganisms that negatively influence the abundance or effectiveness of pathogens. As the physical addition of antagonists has proven to be problematic, changing the playing field by introducing cover crops whose root exudates favor antagonists could be a solution. This report focusses on a selected list of cover crops and will investigate their ability to enhance the abundance of antagonists of *Rhizoctonia solani* (AG2-2 IIIB) and *Pratylenchus penetrans* by means of a pot experiment. In order to study the effect the pathogens have on the cover crops' ability to stimulate antagonists, as well as verifying the antagonistic effect of the antagonists, *R. solani* and *P. penetrans* were inoculated. The data on the pathogens and antagonists, as well as their respective families and phyla, was obtained using quantitative Polymerase Chain Reactions (qPCR's) for which primers were developed *de novo.* Although numerous antagonists were found to be enhanced in the rhizosphere of selected cover crops, no reduction of the abundance of the pathogens could be found. Moreover, the data is compared with previous research in order to discuss the effect of growing conditions on the antagonists, as well as to discuss the comparison of data on absolute abundance to that on relative abundance. All in all, although the data presented in this research may not be ready to use in an agricultural setting just yet, it is a small but sturdy step towards the holy grail of sustainable agriculture.

3. Introduction

Since the 'green revolution' in the 1950's and -60's, agriculture has been dependent on high inputs of fertilizers and pesticides (Y. Liu et al., 2015). Although this high-throughput method caused huge increases of yield over the following decades, it can have detrimental effects on natural ecosystems and biodiversity (Bommarco et al., 2013). As a result, there has been a call from society to farmers to practice a more nature-inclusive form of agriculture during the last couple of decades. However, since a decrease in yield is not wanted, knowledge on how to use ecosystem services in an efficient manner to increase yield is required. Moreover, pesticides have shown to only be effective on pathogens for a limited period of time due pesticide resistance (Hawkins et al., 2019). Therefore, a more 'nature-inclusive' type of pathogen control that can co-evolve with the pathogen could actually be preferable over chemical pesticides.

A large part of a farmer's yield, or lack thereof, is dependent on the level of pathogens within their cropping system. Pathogens can reduce yield by simply damaging the plant or by being vectors for diseases that can negatively influence plant growth. However, not all pathogens are present above the soil-surface. Although a cropping system might seem pathogen-free at the very first glance, soil pathogens are known to have detrimental effects on yield (Fones et al., 2020; Hassan et al., 2013). Moreover, the costs and effort of techniques to combat soil-borne pathogens are high when compared to the management of pests that are present above the surface. In fact more manual labor is required to apply any pesticide or fumigant to the soil (Cao et al., 2019). Secondly, a lot of soil-borne pathogens are hard to combat once the growing season has begun since it would destroy the crop. Lastly, although techniques like fumigation might prove effective against soil borne pathogens, they will also harm the 'good' soil fauna and reduce the biodiversity of the soil ecosystem which can reduce the resilience of the soil (Griffiths et al., 2000). All in all, a novel technique for the reduction of soil-borne pathogens in cropping systems is desired.

Biological control of above-ground pests is relatively well-studied and certain cropping systems simply depend on it (Jacas et al., 2006; van Lenteren et al., 1996). Although biological control of soil-borne pathogens is not as widely implemented as for their above-ground counterpart, it is, in theory, possible. The soil ecosystem on a farm is complex and contains a wide range of microbes, including pathogens, which are for a large part fed by the crop plant. Within this ecosystem, the pathogen will have competition, predation and other types of interaction with soil organisms. Therefore, the presence of a certain soil organism may well inhibit the abundance (or activity) of a pathogen. The diminishing effect a soil can have on pathogen pressure is called the 'suppressiveness' of the soil (Cook, 2014). The suppressiveness of the soil can be subdivided into two distinct categories: general suppression, where the pathogen pressure is reduced by the soil microbiome as a collective, and specific suppression, where pathogen pressure is reduced by a specific (group of) antagonist(s). As a general suppressive soil is the result of long-term agricultural practices which might not be possible in every practical setting, identifying antagonists and including them in the soil microbiome is desired. However, although simply adding beneficial microorganisms, among which antagonists, has proven to be effective from time to time, their effectiveness is, among others, dependent on the ability of the introduced microorganisms to outcompete an already established soil microbiome (Ciancio et al., 2019; Modi & Kumar, 2021; Parnell et al., 2016; Vassilev et al., 2006). Therefore, the direct addition of pathogens' antagonists might not work on every farm. Making use of techniques that could shape the already existing soil microbiome to indirectly enhance more pathogens' antagonists seems to be more fitting. Indirectly shaping the soil microbiome would assure that the antagonists are native and have a chance of persisting under the conditions *in situ.*

Plants are known to shape the microbiome in the rhizosphere, which is likely caused by secreted secondary metabolites that are specific to a family, genus, species or even cultivar of a crop (Jacoby et al., 2020; Lareen et al., 2016; Reinhold-Hurek et al., 2015). Moreover, as a large proportion of the microbiome in the soil is 'dormant' when facing unfavorable circumstances , it may very well be that the plant and its corresponding secondary metabolites play a role in selecting which part of the microbiome is active or dormant (Čihák et al., 2017; Haruta & Kanno, 2015; Lennon & Jones, 2011). All in all, choosing the right plant that shapes the microbiome to include pathogens' antagonist seems like a fitting solution. However, a farmer has to choose a crop because he/she thinks it will generate revenue, not just because it can reduce pathogen pressure. Yet, the choice of cover crops is not as obstinate.

Cover crops are already widely implemented in temperate regions in crop rotation systems as well as 'monocropping' systems. They are planted outside of the growing season to keep the soil from being fallow. Cover crops are planted, among others, to inhibit soil erosion (Langdale et al., 1991), increase the nutrient supply/availability (Hu et al., 1997; Rogers & Giddens, 1957), keep weeds from emerging (Teasdale, 1996), suppress diseases (Wen et al., 2017) and increase organic matter content which affects the structure of the soil and the water/nutrient holding capacity (Nascente et al., 2013; Steenwerth & Belina, 2008). Although cover crops should be selected based on the local environment and the needs of the soil or the following crop, a farmer is relatively free to choose whatever cover crop he wants. Moreover, if the farmer expects yield losses from a specific pathogen in the coming growing season, he could select the cover crop that shapes the microbiome that allows for an increased abundance of antagonists of the expected pathogen, given that the right information is available.

However, selecting the right cover crop is not that simple. Information on the effect of a cover crop on the soil microbiome is scarce, let alone on exactly what pathogens' antagonists are enhanced by the cover crops. Moreover, for a lot of cover crops, it is not even known if they can act as a host for common soil-borne pathogens.

The soil microbiome is a complex and poorly understood ecosystem but, with recent developments in technology, the composition and interactions are starting to be unraveled (Choi et al., 2017; Thakur & Geisen, 2019). High-throughput sequencing has created a relatively unbiased 'lens' to look at the soil microbiome, with the technique improving quickly over time (Rodrigue et al., 2010). Costs of DNA extraction from soil and its sequencing are dropping rapidly as new techniques arise (Bollmann-Giolai et al., 2020; Harkes et al., 2019). However, simply knowing the composition of the microbiome is still a big step away from actually being able to utilize the knowledge in the ever-changing practical situations on farmlands. Therefore, it is important to know which cover crops can enhance a certain antagonist.

Moreover, if one points out a possible increase of antagonist that resulted from usage of a certain cover crop, it is important to note that the antagonist also acts a 'role' in the soil food web. Any antagonistic effect produced by an organism in one place or time might differ from the effect produced by the same organism in another place or time. Therefore, it is important to state how an antagonists' population behaves in a pathogen infected field, or better yet, the reduction of the pathogen during the time the cover crop is present should be included in the research as well.

All in all, cover crops are a promising tool to reduce pathogen pressure by means of enhancing the abundance or activity of antagonists in an organic type of managing. However, at this point in time, it is unknown what cover crop can enhance the abundance or activity of specific species of antagonists. Moreover, the effect these cover crops have on antagonists should be investigated while in proximity of the corresponding pathogens.

3.1. Context of this study

This research can be seen as a follow up study on the work of Cazzaniga (2020; unpublished) and Obinu (2021; unpublished). Summarily, the work by Cazzaniga (2020; unpublished) comprised of growing common Dutch cover crops in 'buckets' (70L pots that were dug into the soil). Afterwards, potatoes were grown on the same soil to find out the cover crops enhanced beneficial microorganisms and to check if those microorganisms remained active after the cover crop had been removed. Throughout different timepoints in the experiment, soil samples were taken and the abundance of the pathogens and the complete microbiome was analyzed by means of high-throughput sequencing. However, since the experiment was free from severe pathogen pressure, no real data on the behavior of antagonists while in proximity of their corresponding pathogens exists as of yet. The work by Obinu (2021; unpublished) comprised of analyzing the sequencing data produced by the experiment of Cazzaniga (2020; unpublished) to see which antagonists were enhanced by which common Dutch cover crops. Since the high-throughput sequencing data was only considered to be reliable up to a family level, no data exists that could pinpoint the antagonists corresponding to the cover crops on a species level at this point in time.

3.2. Verifying the antagonistic effect of the antagonists

Since the original data by Cazzaniga (2020; unpublished) did not include any true pathogen infection, the pot experiment acts as a complimentary experiment to verify that the antagonists enhanced by a cover crop behave in a similar fashion when in presence of a pathogen. Moreover, in order to verify that the selected cover crops will increase the abundance of antagonists, as well as checking whether the antagonists will decrease the abundance of the selected pathogens a pot experiment was conducted.

3.3. Research questions

In order to fill the knowledge gaps presented in this paragraph, the following research questions were named:

- *Which cover crops can shift the microbiome to include more pathogens' antagonists?*
    - *What are the known antagonists of pathogens in Dutch soils?*
    - *What cover crops can enhance these antagonists?*
    - *Can cover crops enhance antagonists even under pathogen pressure?*
- *Does the enhanced presence of antagonists reduce pathogen abundance?*

4. Materials & Methods

4.1. Selecting antagonists and cover crops

The pot experiment included pots filled with soil from the experiment by Cazzaniga (2020; unpublished) that were either inoculated with *Pratylenchus penetrans* or *Rhizoctonia solani* (AG2-2 IIIB). The selection of antagonists was based on the results of Obinu (2021; unpublished) which indicated antagonistic families or genera that were enhanced by one of the cover crops used in the previous experiment by Cazzaniga (2020; unpublished) (table 1). A schematic overview of the pot experiment is shown in Appendix 2 and Figure 7. As the data by Obinu (2021; unpublished) is on a family/genus level, literature was checked for possible antagonists on a species level within the families/genera indicated by Obinu (2021; unpublished). The species within the family/genus indicated by Obinu (2021; unpublished) were selected to be included in this research. As the data that is used to make the selection of antagonists already contains information about the cover crop that has shown to enhance this antagonist, the cover crop selection is fixed to the selection of antagonists.

Table 1. The selection of cover crops that was used by Obinu (2021; unpublished) to search for antagonistic families of P. penetrans and R. solani. Host statuses for P. penetrans and R. solani (AG2-2 IIIB) are included in the table (Aaltjesschema, 2021; Cazzaniga, 2020; unpublished; Van Leeuwen et al., 2019)

Crop	**Common name**	**Family**	**Host status *P. penetrans***	**Host status *R. solani* (AG 2-2)**
Vicia sativa	Common vetch	Fabaceae	Strong host	Unknown
Lens culinaris	Lentil	Fabaceae	Partially resistant	Unknown
Raphanus sativus 'Terranova'	Oilseed radish	Brassicaceae	Strong host	Host
Raphanus sativus 'E1039'	Oilseed radish	Brassicaceae	Resistant (?)	Host
Avena strigosa	Black oat	Poaceae	Non host	Unknown
Festuca arundinacea	Tall fescue	Poaceae	Unknown	Unknown
Lolium hybridum	Hybrid ryegrass	Poaceae	Unknown	Strong host (?)
Phacelia tanacetifolia	Phacelia	Boraginaceae	Strong host	Good host
Borage officinalis	Borage	Boraginaceae	Unknown	Unknown
Tagetes patula	Tagetes	Asteraceae	Active decline	Good/strong host
n.a.	Fallow	n.a.		

4.1.1. Selection of cover crops

The families/genera that were marked antagonistic by Obinu (2021; unpublished) and were selected for this research were Sphingobacteriaceae, Sphingomonadaceae, Pseudomonadaceae, *Acinetobacter spp.,* Aspergillaceae and Cladosporiaceae and were shown to be significantly enhanced in the rhizosphere of respectively borage, black oat, oilseed radish and borage, oilseed radish, lentil and phacelia (appendix 1. figure 1-6). After the literature study it was decided that the antagonistic species/genera that were to be investigated in this research were *Pedobacter spp., Sphingomonas spp.*, *Pseudomonas fluorescens, Pseudomonas trivialis, Acinetobacter calcoaceticus, Aspergillus candidus* and *Cladosporium cladosporioides* belonging to the families/genera of Sphingobacteriaceae, Sphingomonadaceae, Pseudomonadaceae, *Acinetobacter spp.,* Aspergillaceae and Cladosporiaceae respectively. For more information about the selection of cover crops and/or antagonists please see appendix 1.

4.1.2. Setup pot experiment

The experiment was conducted in 3 litre pots, 19cm in diameter and 15cm high. For every cover crop included in the experiment a control without pathogen inoculation was added. Since the experiment included two pathogens treaments, with three cover crops treatment per pathogen, a fallow pot with pathogen/without cover crop, a control without pathogen/with cover crop, a control without pathogen and without cover crop using five replicates, 80 pots in total were used. A schematic overview is shown in figure 7. The 80 pots needed 240 litres of soil (originating from Vreedepeel) to be filled up. The very same soil from a previous experiment by Cazzaniga (2020; unpublished) was dug up and transported to Wageningen University Unifarm, where the experiment was conducted.

The sowing density was chosen to represent a density that reflects realistic farm values which were retrieved from literature or seed trading companies. An overview is given in table 2. The pots where placed in blocks, where each block represented a different batch of Vreedepeel soil. Each treatment was randomly assigned to one of the blocks and randomly placed within the respective group. An overview is given in appendix 2. During the course of the experiment, the soil in the pots were watered regularly.

During the experiment the emergence was checked and extra seeds were sown to when emergence was below 50%. The amount of seeds that were sown was chosen to restore the original sowing density. The crops were given 2.5 months to grow (28-06-2021 until 06-09-2021) after which the rhizosphere was harvested.

Table 2. A table that describes the sowing density in the pot experiment.

**although the calculated required amount of seeds was one seed per pot, it was chosen to sow two seeds per pot to compensate for any dysfunctional seeds*

	1000 seed weight (g)	**Sown per hectare (g/10 m2)**	**sown per pot g/0.028m2**	**seeds/pot**	Reference
borage	19	6.5	0.0182	2*	debolderik.net, janzenzaden.nl
black oat	15	80	0.224	15	(USDA, 2016) agrifirm.nl
oilseed radish	15	35	0.098	7	(Timmer, 2003)
lentil		120 plants/m2		4	(Grownotes, 2018)
phacelia	2.5	10	0.028	11	(Timmer, 2003)

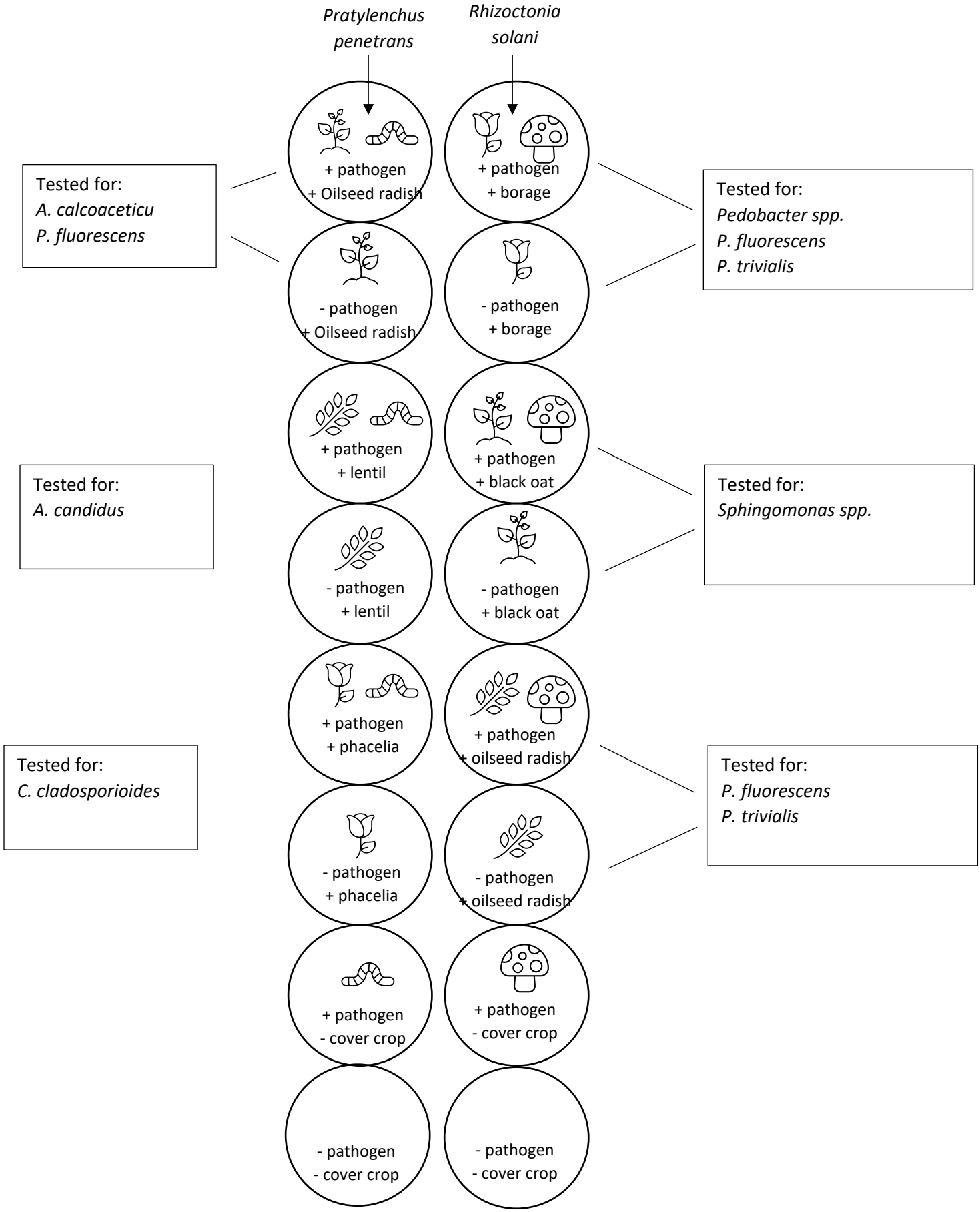


*Figure 1. Schematic overview (**excluding replicates**) of the proposed pot experiment with 2 pathogens and 3 cover crops per pathogen, resulting in 80 pots in total. Although every primer set, including the primer that are specific on a family level was run on every sample. The above figure is merely an overview on why which cover crops and antagonists were selected.*

4.1.2.1. Pathogen inoculation

The pathogens within this experiment were kindly donated by 'WUR open teelten Lelystad' (*Pratylenchus penetrans*) and 'WUR Biointeractions and plant health' (*Rhizoctonia solani* AG2-2 IIIB, in the form of infected oat kernels). The *P. penetrans* was inoculated to the soil at a density of 10 000 per 3L pot, which was considered to be a medium/high level of infection by 'WUR open teelten'.

The inoculation of *R. solani* was done by evenly spreading 20 oat kernels per 3L pot. The cover crops were sown two weeks later in order to give the *R. solani* time to spread through the soil, as was recommended by 'WUR Biointeractions and plant health' and in accordance to Postma and Schilder (2015).

4.1.3. Collecting soil samples

After the pathogens were given two weeks to evenly spread through the soil in the potting experiment and before the sowing of the cover crops, soil samples of approximately 6g were taken by using a small auger to take either one or two cores. The soil samples were stored for later use at -80°C.

After the crops had grown, the rhizosphere of the crops was harvested by shaking off the bulk soil and using a brush to harvest the remaining soil adhering to the plant roots, which was considered to be rhizosphere soil. These soil samples were also stored for later use at -80°C. The cover crops were weighted both before and after being put in a drying oven for three days. At the time of harvest, the soil was visually scored for wetness and were given values of one to four, where one represented the driest samples and four the wettest. Moreover, the plants were visually scored for their health between one and five, where five represented the healthiest individuals and one the most diseased ones.

4.1.4. DNA extraction

Approximately 2g of soil was weighed and noted down after which the protocol as described by Harkes et al. (2019) was used to extract DNA and RNA. The concentrations of both DNA and RNA were measured by using the 'Thermo Fisher Qubit assay' and noted down.

4.2. Quantification of pathogens and antagonists

To be able to quantify the abundance of soil organisms within this research, quantitative Polymerase Chain Reactions (qPCR's) were used. Since qPCR's require primers specific to the target DNA, literature was searched for primers that could be use in this research. Moreover, primers were developed *de novo* in case no primers were found in literature or the ones found performed inadequately.

4.2.1. Selection of antagonists and their respective primers

In order to verify and compare results with the research by Obinu (2021; unpublished), primers were developed that are specific to the antagonistic families/genera listed in appendix 1 and table 3a. Within these families/genera, the literary part of this research uncovered antagonistic species/genera that were to be investigated in this research as well. Therefore, primers were developed for these species/genera too (table 3b). Although some primers for the taxa listed in table 3 were found in literature, it was decided to use primers designed *de novo* after a *in silico* screening of the primers found in literature showed unsatisfactory results.

Besides the primers related to the antagonists, it was decided to use primers specific to bacteria, fungi, *P. penetrans* and *R. solani* as well, which were readily available in the Laboratory of Nematology and the department of Biointeractions and Plan Health at Wageningen University.

Table 3. The list of taxa for which primers are to be developed within this research. a) the taxa for which primers were developed on a species/genus level. b) the taxa for which primers were developed on a family/genus level.

a. Primer construction for:	Abbreviation:
Pedobacter spp.	pedo
P. fluorescens	pf
P. trivialis	pt
Sphingomonas spp.	sm
A. calcoaceticus	acal
A. candidus	acan
C. cladosporioides	c

b. Primer construction for:	Abbreviation:
Pseudomonadaceae	p
Sphingobacteriaceae	sbc
Sphingomonadaceae	smc
Acinetobacter spp.	acine
Aspergillaceae	asp
Cladosporiaceae	cc

4.2.2. Primer design on a species level

The primers corresponding to individual species used in this research were developed by downloading the 16S ribosomal RNA for bacteria or the Internal Transcribed Spacer RNA region for fungi from the 'NCBI nucleotide' database("NCBI Nucleotide," 2021). The downloaded sequences were ran through the 'NCBI Primer-BLAST' which generates primers whilst checking for specificity ("NCBI Primer-BLAST," 2021). Default settings were used with the exception of 'Search mode' (set to: 'User guided'), 'Database' (set to: 'Refseq representative genomes') and 'Organism' (set to either 'Bacteria' or 'Fungi').

As *P. fluorescens* has shown to include a lot of variance within said species, two primer pairs for *P. fluorescens* were developed using the method described in the chapter below: 'Primer design on a genus/family level'. Using this method resulted in primers that picked up more unintended targets but eliminated the chance of developing primer pairs that would not pick up all *P. fluorescens* due to the high variance within that species.

4.2.3. Primer design on a genus/family level

A detailed description of the primer design on a genus/family level can be found in the supplementary material 'protocol specific primer design'. In short, a sequence alignment of the intended target species was either downloaded from the 'LTP' database at ARB-silva or sequences were downloaded from the 'NCBI nucleotide' database and aligned using 'Clustal Omega' after which 'BioEdit sequence alignment editor' was used as the main program to edit and inspect the sequence alignments ("ARB-Silva LTP," 2021; "Clustal Omega," 2021; "NCBI Nucleotide," 2021).

The gaps within the downloaded sequence alignment were removed and the sequences ran through the 'NCBI Primer-BLAST' with the same settings as were used for the primer design for individual species. Although this method will ensure that the resulting primer pairs will pick up all intended targets, it will likely focus on regions within the 16S/ITS that are conserved. Making this method vulnerable to faults within the downloaded sequence data. Since the 'NCBI Primer-BLAST' might skip viable options using this method, the resulting primer pairs were often not specific.

As a way to combat this unspecificity, consensus sequences corresponding the downloaded sequence alignments were made using 'BioEdit' after which the consensus sequence was ran through 'NCBI Primer-BLAST'. The threshold value for the consensus sequences was set at multiple values in the range of 65-98%. A low threshold value will give the 'NCBI Primer-BLAST' more regions as it will not include any degenerate nucleotide bases denoted by the IUPAC degenerate nucleotide code and will therefore result in more specific primer pairs. However, a low threshold value will increase the chance of single nucleotide polymorphism (SNP) mismatches with intended targets, which can be found using 'BioEdit'. These SNP mismatches would have to be resolved by including degenerate nucleotides, a process that will again increase the chance of creating primer pairs that are not specific to just the intended target.

After adding a degenerate nucleotide, the primer pair was ran through the 'NCBI Primer-BLAST' again to re-check their specificity. On the other hand, although a higher threshold value will result in primers that are less likely to have SNP mismatches with intended targets, the possibility to manually check for possibilities to include degenerate nucleotides vanishes since the 'NCBI Primer-BLAST' will not include degenerate nucleotides denoted by the IUPAC code. Because the possibility to manually check for degenerate nucleotides vanishes, 'NCBI Primer-BLAST' might not consider all viable options. Therefore, the best primer pair possible might not be picked up.

4.2.4. Validation of primers *in silico*

The unintended targets that arose with the generated primers were investigated *in silico* and their likeliness to occur in soils with reasonable abundance was estimated. An overview of the designed primers can be found in appendixes 3 and 4. Along with the overview of the designed primers, the tables show unintended targets found with 'NCBI Primer-BLAST' with the settings previously mentioned along with basic information about the unintended targets. If the possibility of an unintended target to occur with reasonable abundance in soil was found, the unintended target was marked as problematic. If the unintended target had a single SNP mismatch to the target, especially when in proximity of the '3 end of the primer, the possibility of using a 'Locked Nucleic Acid' (LNA) was considered if the *in vitro* testing of the primers showed promising results. The melting temperature (Tm) of the primers was evaluated *in silico* using the 'IDT OligoAnalyzer' using default settings with the exception of 'Parameter sets' (set to: 'qPCR') and 'Mg++ Conc' (set to: '5') ("IDT OligoAnalyser," 2021).

4.2.5. Validation of primers *in vitro*

Due to time constraints, a selection was made within the primer pairs that were developed *in silico* to be ordered for *in vitro* testing. The selection was based off an *in silico* validation of the primers as well as the performance of the corresponding crop, the ability to retrieve enough rhizosphere soil and an assessment of the antagonistic potential of the corresponding antagonist. The selection comprised of the following taxa: Pseudomonadaceae, Sphingomonadaceae, Cladosporiaceae, *Acinetobacter spp.*, *P. trivialis, P. fluorescens, Sphingomonas spp., C. cladosporioides* and *A. calcoaceticus*.

In order to test the primers further, cultures of the target species were ordered at 'DSMZ GmbH'. The ordered cultures were DSM 62121, DSM 50090, DSM 1139, DSM 1098, and DSM 14937, corresponding to *C. cladosporioides, P. fluorescens, A. calcoaceticus, Sphingomonas paucimobilis,* and *P. trivialis* respectively. All cultures were received in freeze-dried conditions except DSM 14937 (*P. trivialis*), which was received as an actively growing culture. For additional information on the cultures, please consult the 'DSMZ GmbH' website (www.dsmz.de). The cultures were grown on both the agar plates and the liquid media recommended by 'DSMZ GmbH' for storage and multiplication. The DNA of these target species were extracted using the 'Thermo Fisher GeneJET Genomic DNA Purification Kit', using the gram-negative purification protocol and the yeast purification protocol for respectively the bacterial and fungal taxa. After the DNA purification, the concentration of DNA was measured using the 'Thermo Fisher Qubit' for bacterial taxa and the 'Thermo Fisher Nanodrop' for fungal taxa. The differentiation between these types of measuring was made because the DNA of the fungal taxa was too heavily contaminated for Qubit measurements.

The annealing temperature of the primers was assessed by using a qPCR with a temperature gradient ranging from 55°C to 65°C. The optimal annealing temperature was selected by choosing the temperature where the primer efficiency was the highest. For every target taxa, one or two primer pairs were selected for further assessment based on a combination of a high primer efficiency and the related melt curve having a single parabolic shape. The primer pairs selected for further assessment were: acine-1, smc-1, c-3, p-3, cc-1, cc-3, pf-2, pf-4, acal-1, pt-1, sm-2, and sm-3. Additional information on these primer pairs can be found in appendixes 3 and 4.

The selection of primers was tested further by running them on a qPCR with the extracted DNA of the cultures of their respective target species diluted in a ten-fold series along with a negative control (a qPCR-well without target DNA added). Since the starting concentrations of DNA of the dilution series are known, and thus all the DNA concentrations within the series, the quantitative cycle (Cq) value that results from the qPCR can be directly linked to a concentration of DNA. The logarithms of the known DNA concentrations of target species were plotted against their respective Cq values that resulted from the qPCR. A regression line was fitted which was later used to calculate the concentrations of sample DNA, as well as being able to calculate the efficiency of the primers. Cq values that deviated notably from the fitted regression line were considered to be due to pipet errors or sample contamination and were therefore discarded. The efficiencies along with the results of the negative controls were used to make a final selection of the primer pairs that were to be used on the samples. The selection of primer pairs that were used on the samples was: acine-1, smc-1, c-3, p-3, cc-3, pf-4, acal-1, pt-1, and sm-3. Please note that only the cultures related to the primer pairs designed *de novo* were available. Therefore, it was not possible to use the Cq values that resulted from the primers that were available in the Nematology laboratory (universal bacteria and fungi, and *P. penetrans*) to calculate an exact concentration of DNA. The listed concentrations of DNA that were retrieved using these primer pairs should therefore merely be seen as an approximation.

In order to test the specificity of the primer *in vitro*, the products that were amplified during qPCR's of sample DNA or the DNA of pure cultures were run on 1.5% agarose gel to assess the number of base-pairs of the qPCR products. The number of base-pairs were compared to the theoretical number of base-pairs that was found in the *in silico* testing of the primers. Moreover, if the gel showed multiple bands or smears, the primers were considered less specific.

4.2.6. Conducting the qPCR's

The DNA that was extracted from the soil samples was diluted 10 times to minimize inhibition that arises from sample contamination. The dilutions were analysed by using qPCR with the threshold cycle (Ct) set at 80 for all samples. Any well within the qPCR plate was filled with 5µL of sterilized Milli-Q water, 10µL of Sybr-Green IQ mix, 2µL the respective 10-fold diluted primer pair, and 3µL of the respective sample.

The qPCR related to *R. solani* was conducted in cooperation with Carin Lombears (WUR, biointeractions and plant health). Other than all other qPCR's used in this experiment, the qPCR related to *R. solani* was a Taqman qPCR as opposed to the Sybr-Green qPCR. Similar to the primers designed *de novo*, a dilution series of known concentrations of DNA was used to be able to calculate the exact concentration of DNA in the qPCR-well.

Within this research, the amount of DNA measured by a qPCR is considered to be directly connected to the abundance of the respective taxa.

4.2.7. Calculating the concentration of DNA

Using the regression line that resulted from running the primers with a 10-fold dilution series the concentration of DNA was calculated. The formula of the regression line was y=ax+b, where y=the Cq value, a=the value with which the Cq value increases when the logarithm of the DNA concentration increases by one, x=the logarithm of the DNA concentration, and b=the Cq value when x=0. In order to calculate the DNA concentrations in any of the qPCR's equation 1 was used, which is derived from the regression line that resulted from the dilution series. Moreover, the calculated concentration was corrected for the amount of soil that was used to extract DNA from (approximately 2 grams) and for any dilutions that occurred during the DNA extraction or qPCR's.

$$DNA\ concentration = 10^{\frac{y-b}{a}} \qquad \textit{Equation 1.}$$

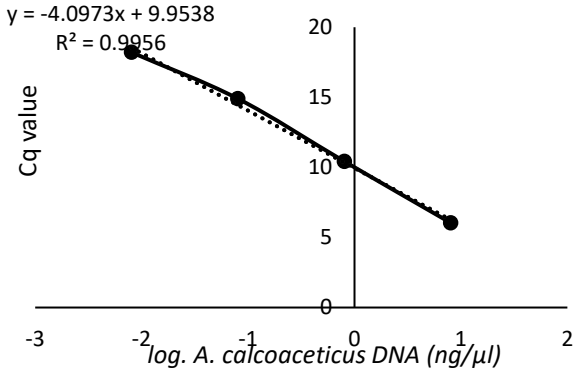
4.3. Statistical analysis

All data was analysed using 'IBM SPSS statistics v.27'. Any datapoint that showed a deviating melt curve in the qPCR analysis, or for which the harvest of rhizosphere soil was faulty were left out of the statistical analysis completely. Moreover, when a pipet error occurred during the loading of the qPCR plate, the related sample was left out of the statistical analysis as well, an overview of which samples have been left out of the analysis can be found in the attachment 'Included Samples'. Differences between sample groups were considered to be significant when $p<=0.05$. All data related to qPCR's within this experiment did not meet normality assumptions and will therefore be analysed using non-parametric tests. Besides the data that resulted from the qPCR, the data on the growth conditions (e.g. soil wetness, batch of soil) were tested for their influence on the abundance of any of the taxa. The statistical tests used in this research are: 1) the Kruskal-Wallis 1-way ANOVA, which is used to test for a change in abundance of any of the taxa at the same point in time as a result of a treatment, 2) if a significant value is found by the Kruskal-Wallis 1-way ANOVA, the Bonferroni correction is used for multiple comparison between treatments, as well as the comparison of treatment – control, 3) the Wilcoxon matched-pair signed-rank is used to compare the pre-sowing time point to the harvest time point.

5. Results

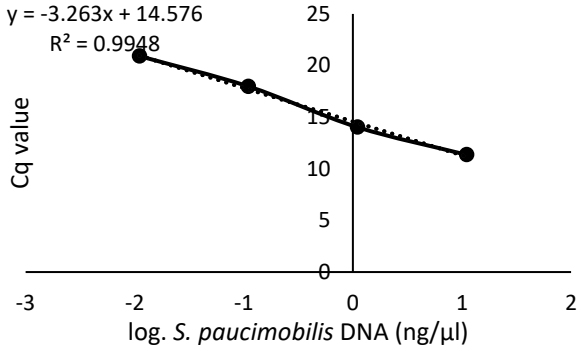
5.1. Primer testing

In the figures 8 to 16, the Cq values related to the 10-fold dilution series of the DNA of the pure cultures can be found, as plotted against the logarithmic value of the known concentration of DNA. The undiluted DNA concentrations were 8.0, 11.0, 14.0, 4.2 and 4.0 ng/µl for the cultures of *A. calcoaceticus, S. paucimobilis, P. fluorescens, C. cladosporioides,* and *P. trivialis*, respectively.



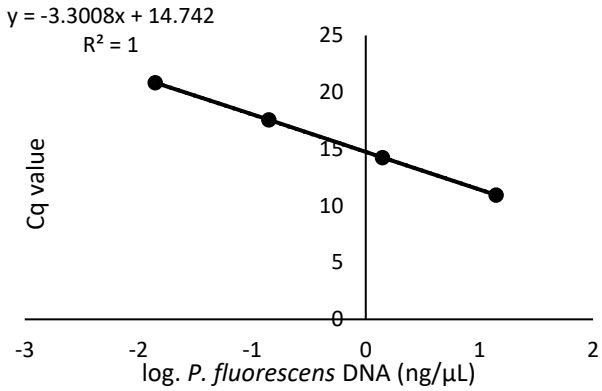
Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
8.0	0.903	6.048
0.80	-0.097	10.431
0.080	-1.097	14.906
0.0080	-2.097	18.214

Figure 2. Results related to the qPCR of primer pair acine-1 when run with a 10-fold dilution series of A. calcoaceticus DNA of known concentrations. The fitted regression line yielded the formula y=-4.0973x+9.9538 and has a squared residual value of 0.9956, the corresponding primer efficiency is 75.4%. The qPCR was run with the optimal annealing temperature of acine-1, which was 59°C.



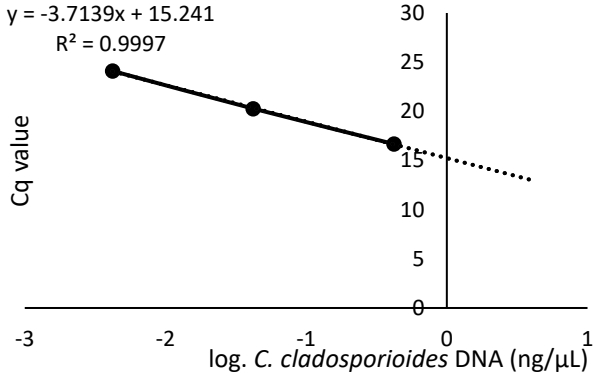
Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
11.0	1.041	11.359
1.10	0.041	14.032
0.110	-0.959	17.982
0.0110	-1.959	20.919

Figure 3. Results related to the qPCR of primer pair smc-1 when run with a 10-fold dilution series of S. paucimobilis DNA of known concentrations. The fitted regression line yielded the formula y=-3.263x+14.576 and has a squared residual value of 0.9948, the corresponding primer efficiency is 102.5%. The qPCR was run with the optimal annealing temperature of smc-1, which was 59°C.



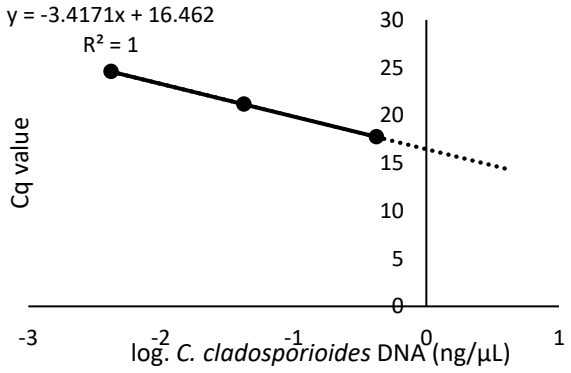
Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
14.0	1.146	10.950
1.40	0.146	14.261
0.140	-0.854	17.582
0.0140	-1.854	20.846

Figure 4. Results related to the qPCR of primer pair p-3 when run with a 10-fold dilution series of P. fluorescens DNA of known concentrations. The fitted regression line yielded the formula y=-3.3008x+14.742 and has a squared residual value of 1.0000, the corresponding primer efficiency is 100.9%. The qPCR was run with the optimal annealing temperature of p-3, which was 59°C.



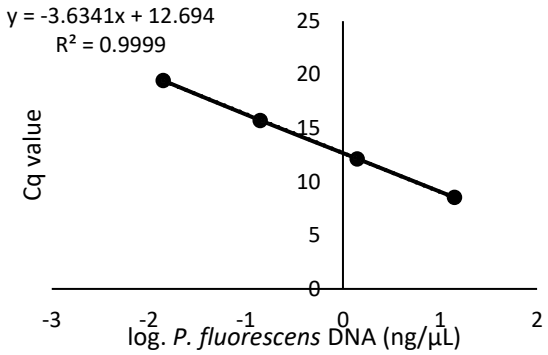
Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
4.2	0.623	n/a
0.42	-0.377	16.677
0.042	-1.377	20.280
0.0042	-2.377	24.104

Figure 5. Results related to the qPCR of primer pair c-3 when run with a 10-fold dilution series of C. cladosporioides DNA of known concentrations. The fitted regression line yielded the formula y=-3.7139x+15.241 and has a squared residual value of 0.9997, the corresponding primer efficiency is 85.9%. The qPCR was run with the optimal annealing temperature of c-3, which was 63.3°C.



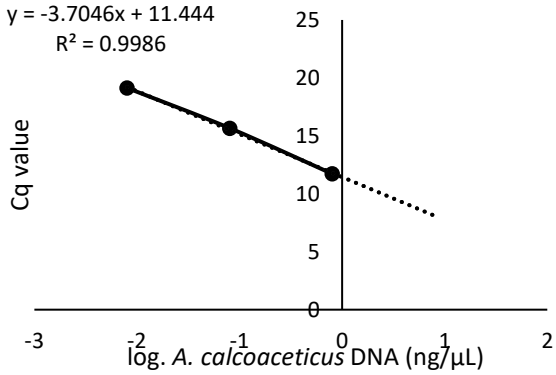
Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
4.2	0.623	n/a
0.42	-0.377	17.751
0.042	-1.377	21.162
0.0042	-2.377	24.585

Figure 6. Results related to the qPCR of primer pair cc-3 when run with a 10-fold dilution series of C. cladosporioides DNA of known concentrations. The fitted regression line yielded the formula y=-3.4171x+16.462 and has a squared residual value of 1.000, the corresponding primer efficiency is 96.2%. The qPCR was run with the optimal annealing temperature of cc-3, which was 65°C.



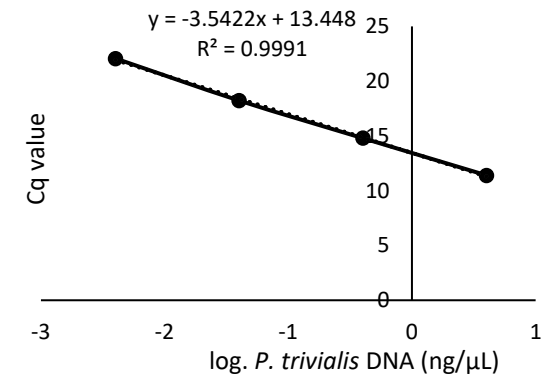
Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
14.0	1.146	8.547
1.40	0.146	12.161
0.140	-0.854	15.746
0.0140	-1.854	19.466

Figure 7. Results related to the qPCR of primer pair pf-4 when run with a 10-fold dilution series of P. fluorescens DNA of known concentrations. The fitted regression line yielded the formula y=-3.6341x+12.694 and has a squared residual value of 0.9999, the corresponding primer efficiency is 88.4%. The qPCR was run with the optimal annealing temperature of pf-4, which was 65°C.



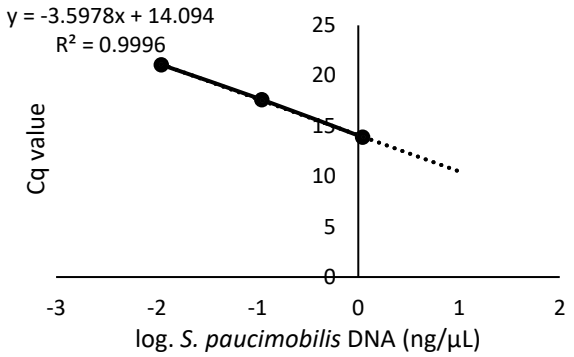
Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
8.0	0.903	n/a
0.80	-0.097	11.724
0.080	-1.097	15.666
0.0080	-2.097	19.133

Figure 8. Results related to the qPCR of primer pair acal-1 when run with a 10-fold dilution series of A. calcoaceticus DNA of known concentrations. The fitted regression line yielded the formula y=-3.7046+11.444 and has a squared residual value of 0.9986, the corresponding primer efficiency is 86.2%. The qPCR was run with the optimal annealing temperature of acal-1, which was 65°C.



Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
4.0	0.602	11.410
0.40	-0.398	14.800
0.040	-1.398	18.234
0.0040	-2.398	22.073

Figure 9. Results related to the qPCR of primer pair pt-1 when run with a 10-fold dilution series of P. trivialis DNA of known concentrations. The fitted regression line yielded the formula y=-3.5422x+13.448 and has a squared residual value of 0.9991, the corresponding primer efficiency is 86.2%. The qPCR was run with the optimal annealing temperature of pt-1, which was 65°C.



Concentration of culture DNA (ng/ul)	Logarithm of DNA concentration	Measured Cq value
11.0	1.041392685	n/a
1.10	0.041392685	13.90114855
0.110	-0.958607315	17.62989613
0.0110	-1.958607315	21.09670513

Figure 10. Results related to the qPCR of primer pair sm-3 when run with a 10-fold dilution series of S. paucimobilis DNA of known concentrations. The fitted regression line yielded the formula y=-3.5978x+14.094 and has a squared residual value of 0.9996, the corresponding primer efficiency is 89.6%. The qPCR was run with the optimal annealing temperature of sm-3, which was 65°C.

5.2. Verification of the pathogen inoculation

5.2.1. *R. solani*

The samples that were inoculated with the *R. solani* kernels showed a significantly higher abundance of *R. solani* DNA when compared to the samples that were not inoculated with the pathogen at the pre-sowing time point (*p<0.001*; Kruskal-Wallis 1-way ANOVA adjusted by the Bonferroni correction; figure 17).

5.2.2. *P. penetrans*

The samples that were inoculated with *P. penetrans* did not show a significant difference in the abundance of *P. penetrans* DNA when compared with the samples that were not inoculated with the pathogen for both the pre-sowing time point as well as at the harvest time point (Kruskal-Wallis 1-way ANOVA).

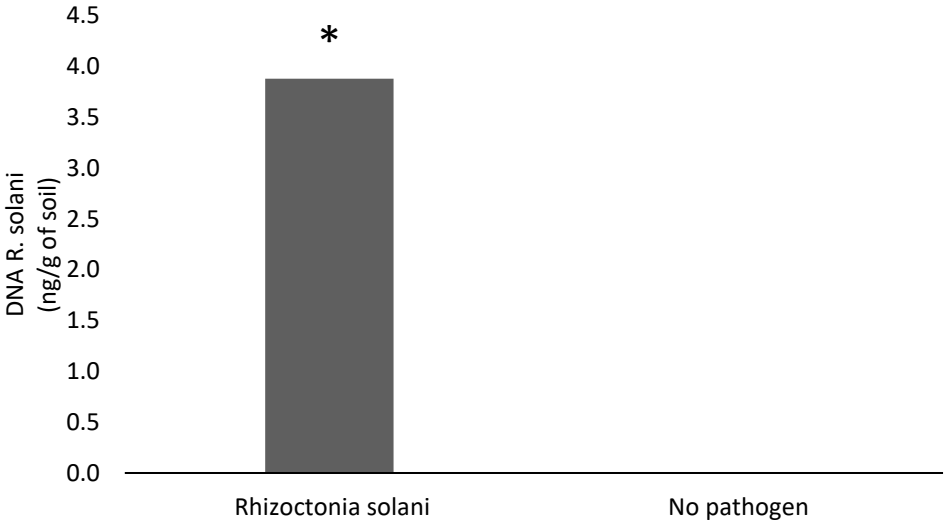


*Figure 11. The abundance of R. solani in the R. solani treated pots and the untreated controls before any crops were sown. Significance indicated by **

5.3. Sphingomonadaceae and their respective antagonist(s)

The abundance of Sphingomonadaceae DNA was significantly enhanced when comparing the rhizosphere soil at harvest time to the soil before the seeds were sown (*p=0.007*; Wilcoxon matched-pair signed-rank; figure 18). The abundance of Sphingomonadaceae was not significantly influenced by pathogen inoculation (Kruskal-Wallis 1-way ANOVA) or by cover crop when comparing the abundance of rhizosphere soil at harvest time to the fallow control (Kruskal-Wallis 1-way ANOVA).

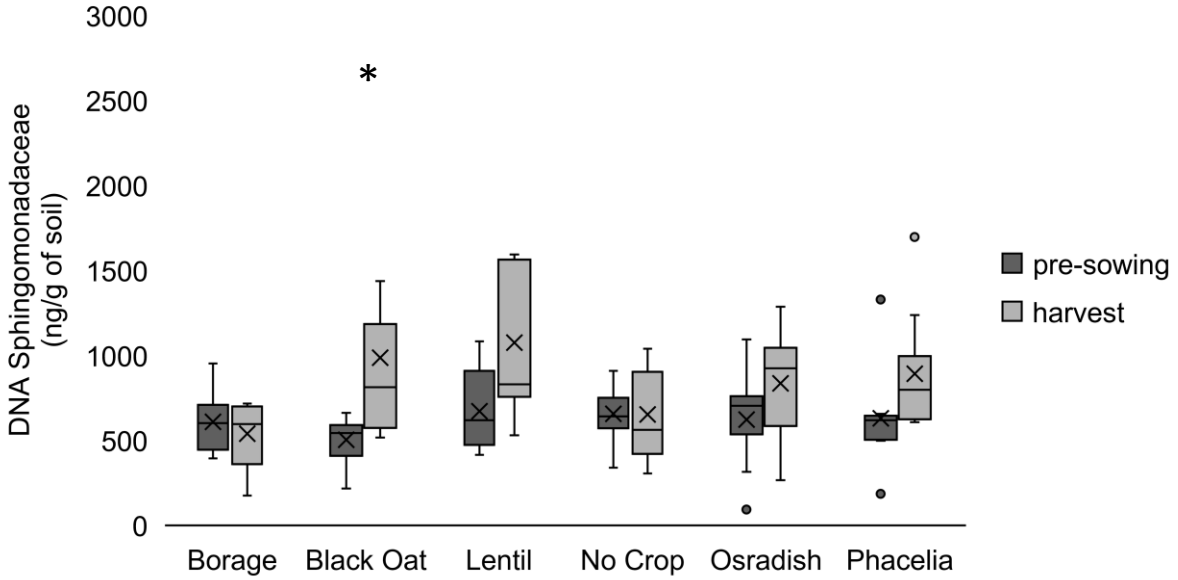


*Figure 12. Abundance of Sphingomonadaceae in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

The abundance of the antagonistic genus *Sphingomonas* is significantly enhanced in the rhizosphere of black oat, lentil and oilseed radish when compared to the soil at the time of sowing (respectively *p=0.028, p=0.017 & p=0.027;* Wilcoxon matched-pair signed-rank; figure 19). The pathogen inoculation as well as the cover crop treatment when compared to the no crop control did not yield any significant results (Kruskal-Wallis 1-way ANOVA).

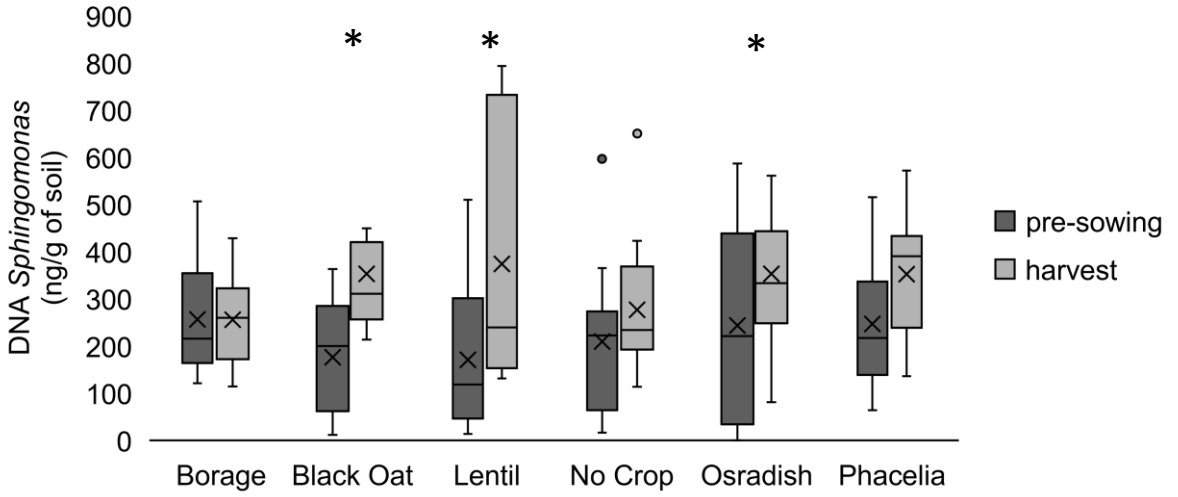


*Figure 13. Abundance of Sphingomonas in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

5.4. Cladosporiaceae and their respective antagonist(s)

No significant effect was measured as a result of pathogen inoculation (Kruskal-Wallis 1-way ANOVA). However, in proximity of both black oat and oilseed radish the Cladosporiaceae were significantly enhanced when compared to the same soil before sowing (respectively *p=0.009* & *p<0.001;* Wilcoxon matched-pair signed-rank) as well as compared to the fallow control (respectively *p=0.009* & *p=0.015*; Kruskal-Wallis 1-way ANOVA adjusted by the Bonferroni correction; figure 20).

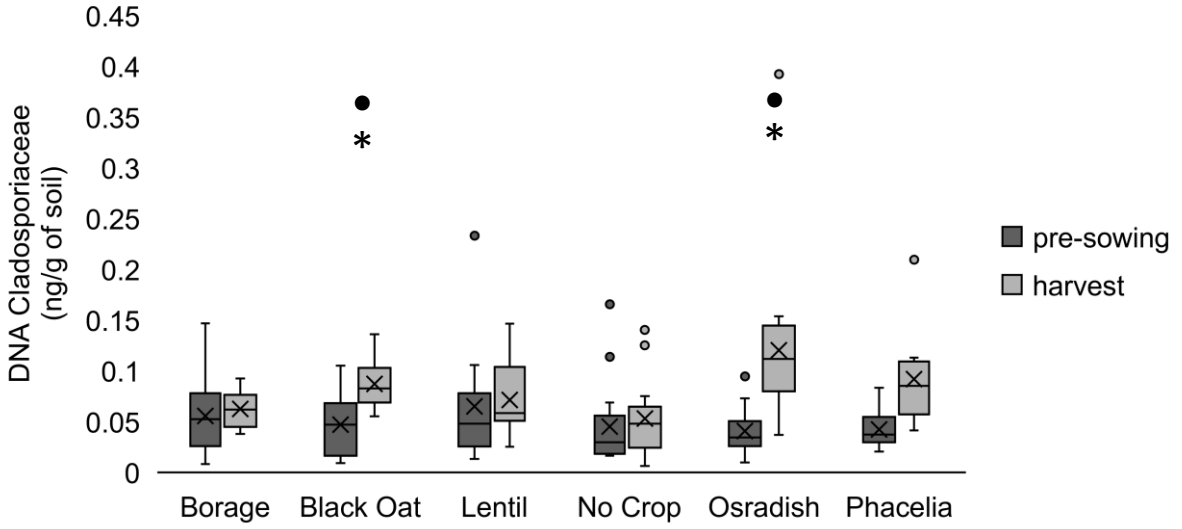


*Figure 14. Abundance of Cladosporiaceae in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

The abundance of *C. cladosporioides* was significantly affected by the inoculation of pathogens (*p=0.030,* Kruskal-Wallis 1-way ANOVA), although no pairwise significant differences were found when adjusted for the Bonferroni correction. The abundance of *C. cladosporioides* was significantly enhanced in the rhizosphere of lentil when compared with the same soil before sowing (*p=0.028*; Wilcoxon matched-pair signed-rank; figure 21).

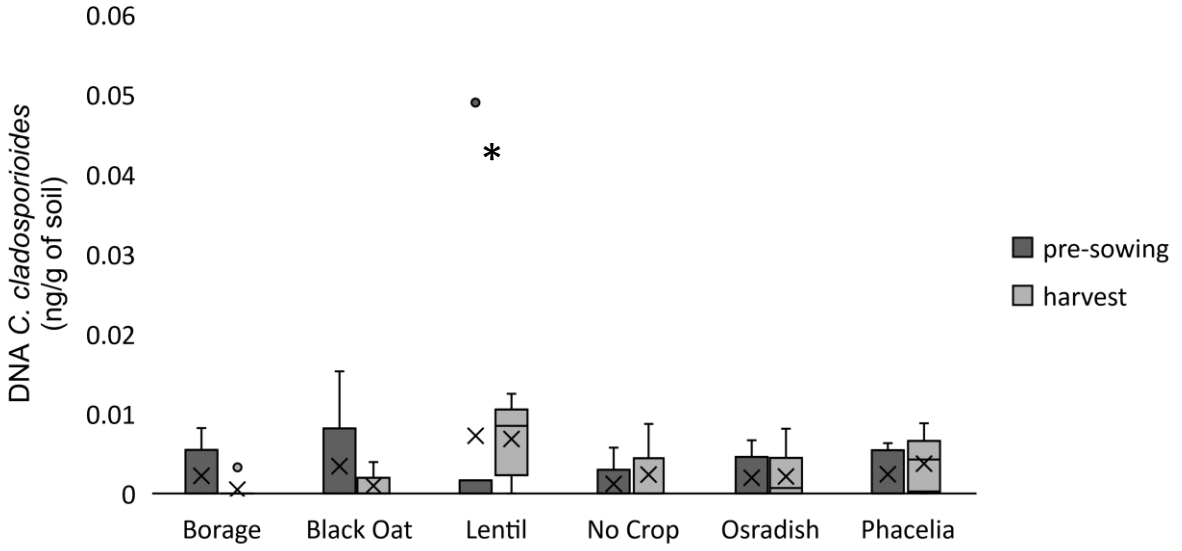


*Figure 15. Abundance of C. cladosporioides in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

5.5. Pseudomonadaceae and their respective antagonist(s)

The abundance of the Pseudomonadaceae was not found to be significantly affected by the rhizosphere of cover crops when compared to the fallow control (Kruskal-Wallis 1-way ANOVA). However, compared to the pre-sowing time point, the Pseudomonadaceae abundance decreased significantly in the rhizosphere of borage, while the abundance increased in the rhizosphere of black oat (respectively *p=0.005* & *p=0.017*; Wilcoxon matched-pair signed-rank; figure 22). Inoculation of pathogens did not yield any significant difference in the abundance of Pseudomonadaceae (Kruskal-Wallis 1-way ANOVA).

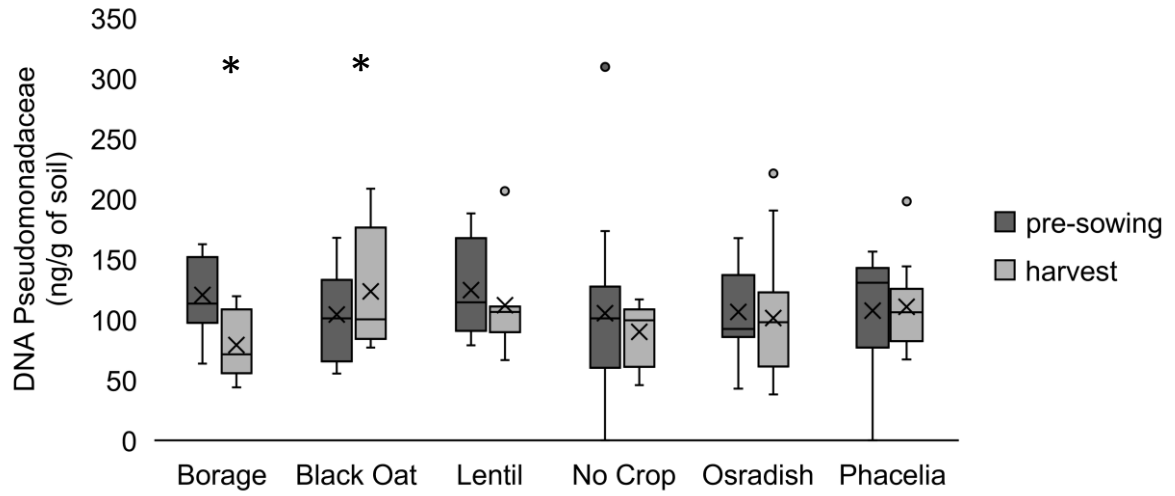


*Figure 16. Abundance of Pseudomonadaceae in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

The abundance *P. trivialis* was significantly enhanced in the rhizosphere of black oat when compared to the same soil before sowing (*p=0.037*; Wilcoxon matched-pair signed-rank; figure 22). The abundance of *P. fluorescens* was significantly enhanced when in proximity of lentil roots compared to the same soil before sowing (*p=0.022*; Wilcoxon matched-pair signed-rank; figure 23). For the abundance of both *P. trivialis* and *P. fluorescens,* no significant differences were found when they were compared to the fallow control or as a result of pathogen inoculation (Kruskal-Wallis 1-way ANOVA).

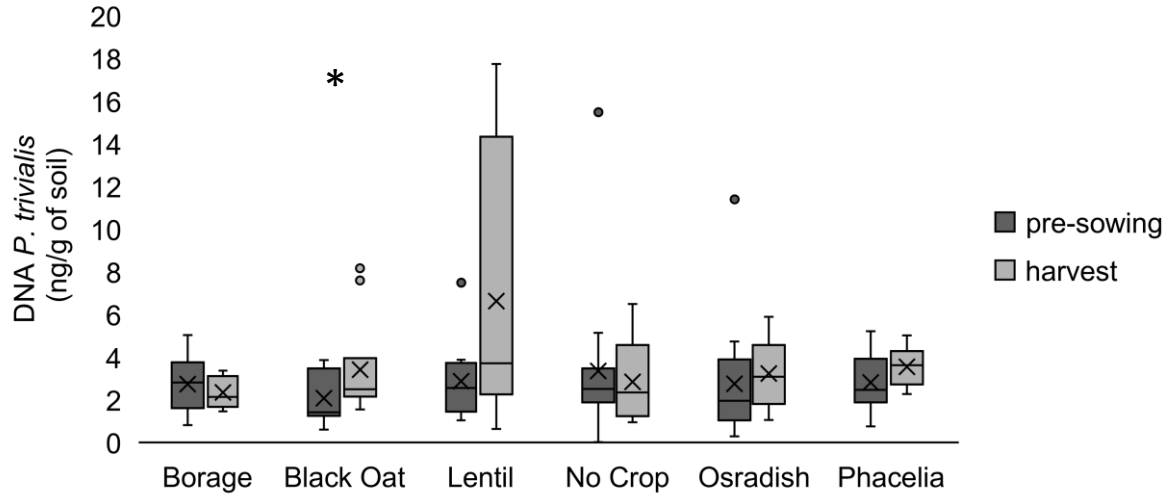


*Figure 17. Abundance of P. trivialis in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

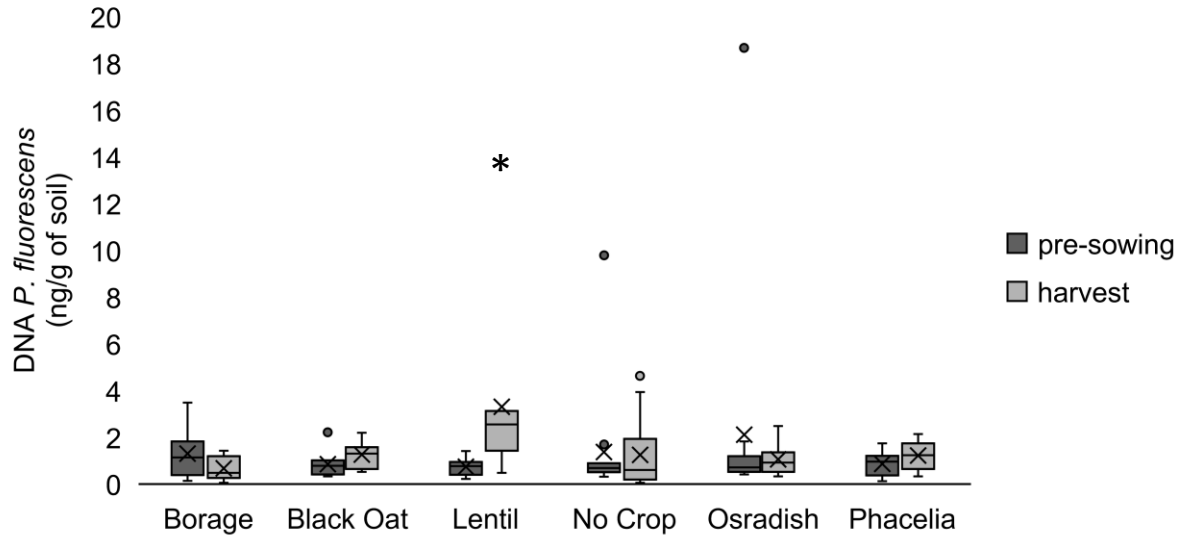


*Figure 18. Abundance of P. fluorescens in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

5.6. Acinetobacter and their respective antagonist(s)

No significant differences were found that were due to the inoculation of pathogens or when rhizosphere soil was compared to the bulk soil of the fallow control (Kruskal-Wallis 1-way ANOVA). The fallow control as well as the phacelia rhizosphere soil showed a significant decrease in the abundance of the genus *Acinetobacter* (respectively *p=0.041* & *p=0.005;* Wilcoxon matched-pair signed-rank; figure 25). As for *A. calcoaceticus*, no usable data could be generated using qPCR with any of the primer pairs due to a contamination of some sort. Therefore, no results on *A. calcoaceticus* could be displayed.

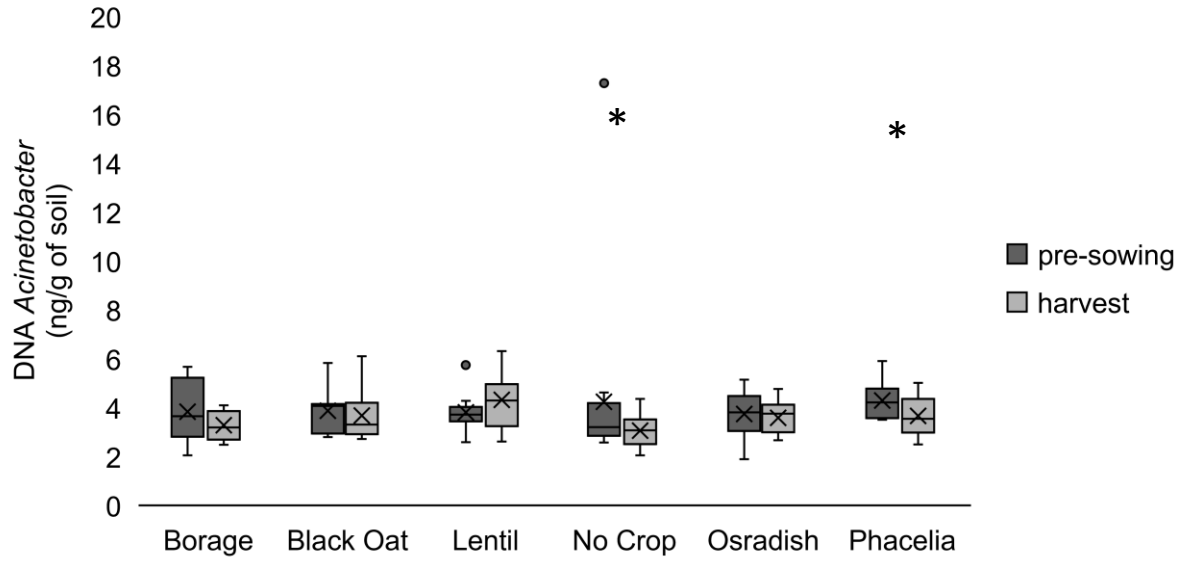


*Figure 19. Abundance of Acinetobacter in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

5.7. Bacteria & fungi

All cover crops showed an increased amount of bacteria in their rhizosphere when compared to the same soil before the crops were sown (*p=0.009, p=0.005, p=0.045, p<0.001* & *p=0.013* for respectively borage, black oat, lentil, oilseed radish and phacelia; Wilcoxon matched-pair signed-rank; figure 26). None cover crops' respective rhizospheres showed a significant difference when as a result of the cover crop or as result of pathogen inoculation (Kruskal-Wallis 1-way ANOVA). In the case of fungal abundance, both black oat and oilseed radish showed a significant increase (respectively *p=0.022* & *p<0.001;* Wilcoxon matched-pair signed-rank) compared to the same soil before sowing. Similarly, the oilseed radish showed significantly more fungi when compared to the fallow control (*p<0.001,* Kruskal-Wallis 1-way ANOVA adjusted by the Bonferroni correction; figure 27).

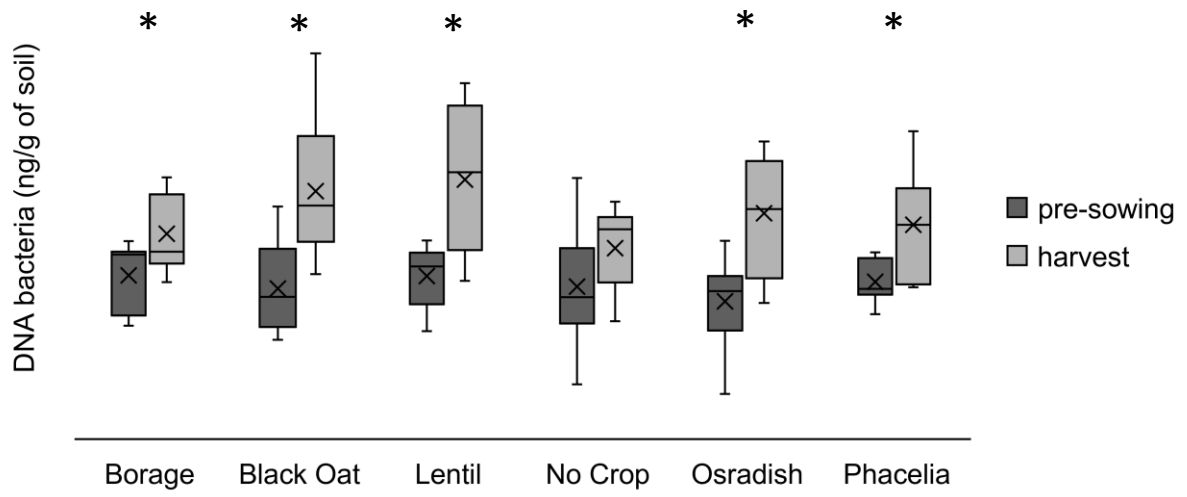


*Figure 20. Abundance of bacteria in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

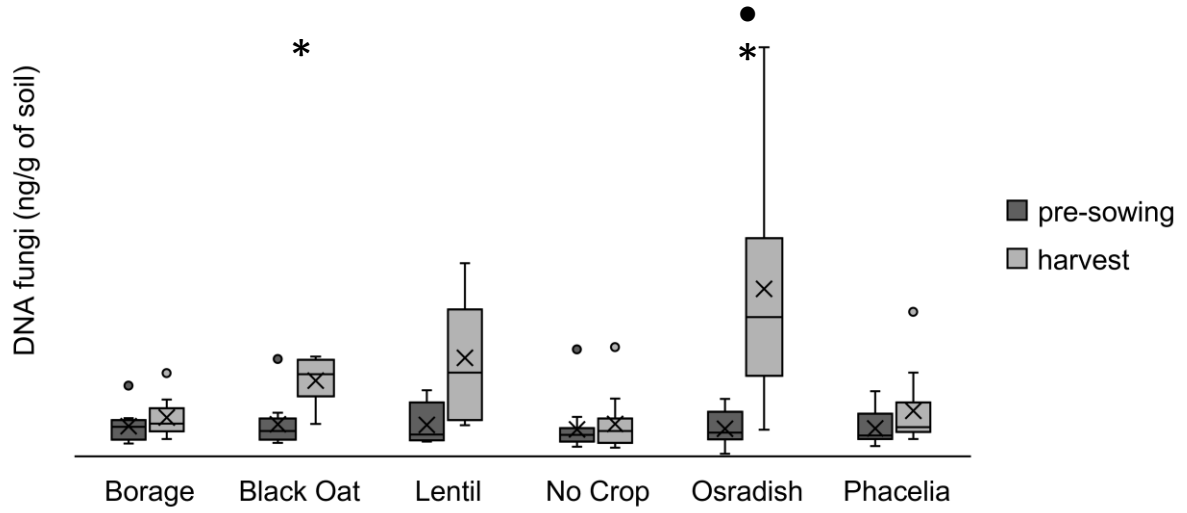


*Figure 21. Abundance of fungi in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *.*

5.8. Verifying the specificity of the primer pairs

Although the primer pairs were tested *in silico* for specificity, the amplicons that were produced during the qPCR's were run on gel and compared with the values that were obtained from the *in silico* testing (figure 28). The amplicons related to *A. calcoaceticus, P. trivialis, P. fluorescens* and *Sphingomonas* approximated their theoretical values of respectively 740, 263, 575 and 600 base pairs. However, the *C. cladosporioides* product did not approximate its theoretical 341 base pairs, even in case of the positive control, which consists of only diluted *C. cladosporioides* DNA. Moreover, the gel shows a similar band for *A. calcoaceticus* DNA when comparing the negative control (without added DNA) to the samples with DNA, indicating a contamination of some sort.

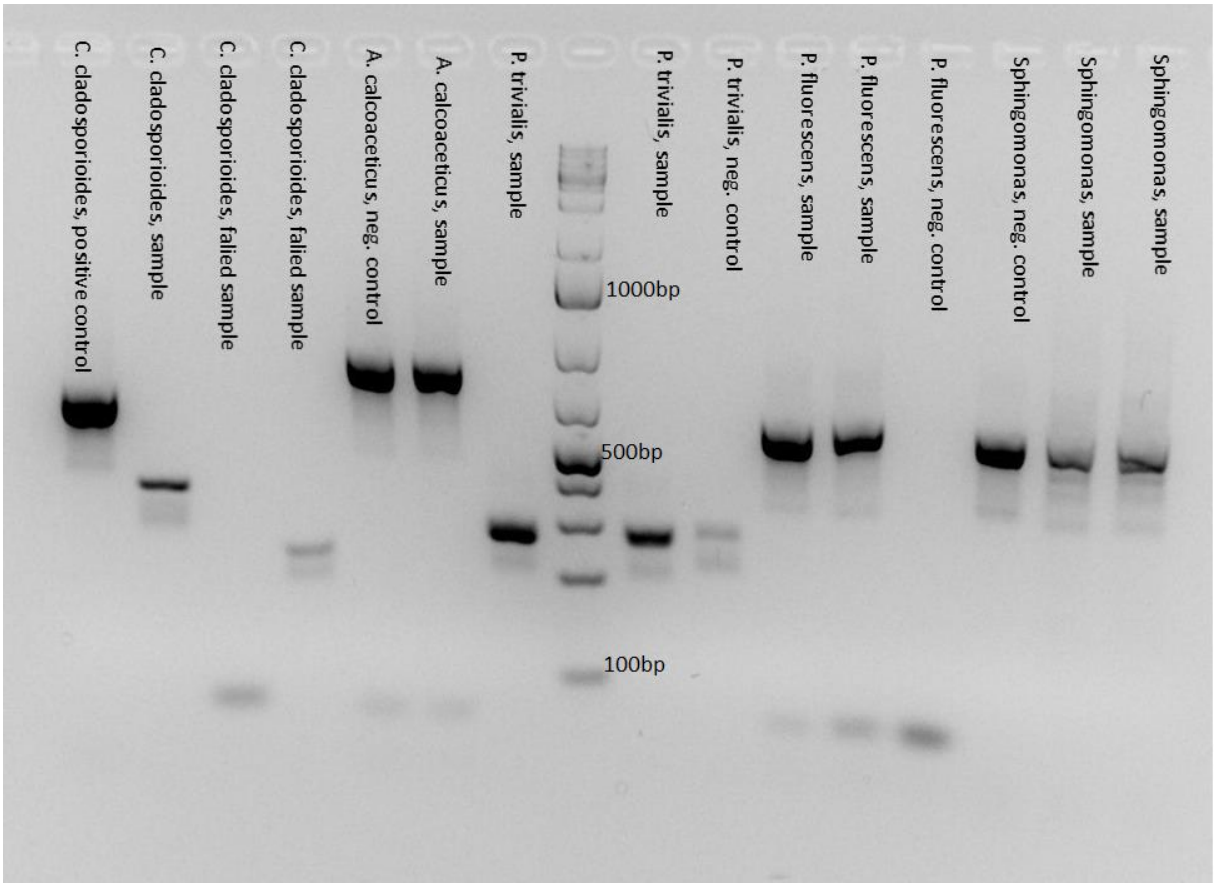


Figure 22. A photo of the 1.5% agarose gel loaded with PCR products that arose from qPCR's that contained samples (as indicated by 'sample'), extracted DNA of a pure culture of the target organism (as indicated by 'positive control') or sterilized Milli-Q water (as indicated by 'neg. control').

5.9. Pathogen reduction as a result of cover crops

The abundance of *P. penetrans* was not significantly affected by cover crops when compared to the fallow control (Kruskal-Wallis 1-way ANOVA) or when comparing the rhizosphere soil to the pre-sowing bulk soil (Wilcoxon matched-pair signed-rank). Similar to *P. penetrans*, the abundance of *R. solani* was not significantly affected by the cover crops when compared to the fallow control or the pre-sowing time point (Kruskal-Wallis 1-way ANOVA; Wilcoxon matched-pair signed-rank). However, a significant reduction of *R. solani* abundance had taken place in the fallow control (*p=0.028;* Wilcoxon matched-pair signed-rank; figure 29).

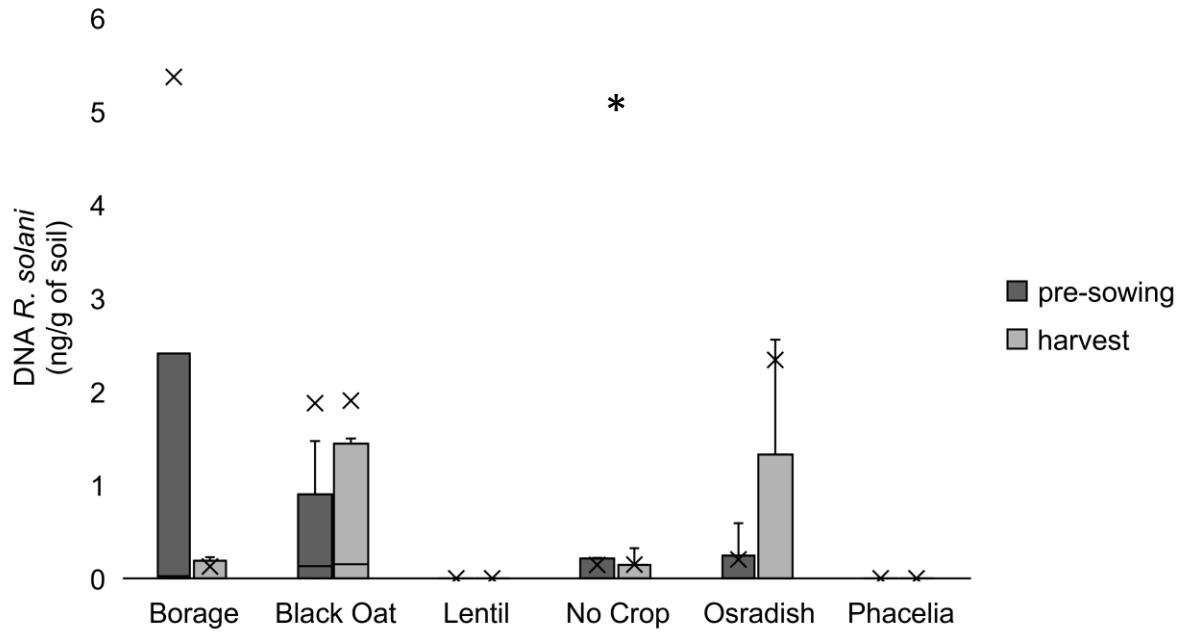


*Figure 23. Abundance of R. solani in pre-sowing bulk soil or rhizosphere soil of selected cover crops at harvest time. Significant differences of the rhizosphere soil at harvest compared to no crop control at harvest are denoted by •, significant differences compared to same soil before sowing are denoted by *. Please note that the outliers were removed from this graph to improve readability.*

5.10. Additional data

When comparing the differences of visually scored soil moisture contents as a factor at harvest time, it shows that it significantly influences the abundance of bacteria, fungi, Pseudomonadaceae, Cladosporiaceae and Sphingomonadaceae (respectively *p=0.018, p<0.001, p=0.009, p=0.009*, p=0.018*;* Kruskal-Wallis 1-way ANOVA).

Moreover, the abundances of bacteria, *P. penetrans,* Pseudomonadaceae, *C. cladosporioides, P. fluorescens* were significantly affected at harvest time as a result of the batch of soil that was used (respectively *p<0.001, p=0.005, p=0.028* & *p=0.003;* Kruskal-Wallis 1-way ANOVA).

5.11. Individual data

All data used in this experiment, including data which has not been mentioned in the results section of this research (e.g. dry weight of the plant, primer dilution schemes of primer pairs that were not used in this research) can be found in the supplementary file 'data'.

6. Discussion

6.1. Comparison of the results

As mentioned in the introduction chapter of this paper, this research was conducted to act as a complementary research to (Cazzaniga, 2020; unpublished) and (Obinu, 2021; unpublished). As their results about the relative abundance were on a family or genus level, those same families or genera have been tested for the absolute abundance in this very research.

The relative abundance of the Sphingomonadaceae was significantly enhanced compared to the fallow control as well when compared to the bulk soil as was shown by Obinu (2021; unpublished). Similarly, the abundance of the Sphingomonadaceae in this research was significantly enhanced when compared to the bulk soil, albeit not compared to the fallow control. Moreover, in the case of *Sphingomonas spp.,* the genus was enhanced when in the rhizosphere of black oat, which was to be expected based on the results of Sphingomonadaceae in this research and the one by Obinu (2021; unpublished). However, in the report by Obinu (2021; unpublished), the relative abundance of the Sphingomonadaceae decreased when in the rhizosphere of oilseed radish, which was not the case in this experiment. In literature, no results were found that confirmed the *Sphingomonas spp.* – black oat association. However, Maglione et al. (2021) showed that the *Sphingomonas* were promoted by rye as cover crop, while Chinta et al. (2021) showed an unsignificant decrease of *Sphingomonas* as a result of cover crop.

In the research by Obinu (2021; unpublished), the relative abundance of the Cladosporiaceae was significantly enhanced in the presence of, among others: borage, black oat, lentil, and when considering cDNA; phacelia as well. Likewise, in this experiment, the Cladosporiaceae were significantly enhanced in the rhizosphere of black oat when compared to bulk soil as well as the fallow control. However, this was not the case for borage, lentil or phacelia as was expected based on the previous research. On a species level, *C. cladosporioides* was shown to be enhanced in lentil rhizosphere, which corresponds with the results by Obinu (2021; unpublished), but not with the results of this research. No papers were found in literature that described the (relative) abundance of *C. cladosporioides* as a result of cover crops.

The Pseudomonadaceae family did not behave as expected when comparing this research to Obinu (2021; unpublished). While Obinu (2021; unpublished) stated that the relative abundance of Pseudomonadaceae was significantly enhanced in the presence of borage, this research actually shows a decrease of Pseudomonadaceae in the rhizosphere of borage. Moreover, the increase of Pseudomonadaceae in oilseed radish rhizosphere by Obinu (2021; unpublished) was also not found in this study. In addition, the increase of Pseudomonadaceae in the rhizosphere of black oat found in this research, was not visible in Obinu (2021; unpublished). However, Patkowska (2020) found that *Pseudomonas spp.* was significantly enhanced compared to a fallow control in the rhizosphere of oat (*Avena sativa*). Although the results by Patkowska (2020) are not directly comparable, it does indicate that (members of) the genus *Pseudomonas* are enhanced by black oat rhizosphere. Similarly, *P. trivialis* was shown to be enhanced in the rhizosphere of black oat in this research as well. Both the abundance of *P. trivialis* and *P. fluorescens* did not behaved as expected based on Obinu (2021; unpublished).

As for *Acinetobacter*, no significant increase was found in this research as a result of cover crops or pathogen inoculation which is different than the research by Obinu (2021; unpublished), where the relative abundance was significantly enhanced for oilseed radish.

When comparing this research to the research that was conducted by Obinu (2021; unpublished) or Cazzaniga (2020; unpublished), it is important to keep in mind that the setup of the research is different, as well as that the very data measured (abundance vs relative abundance) cannot be compared directly.

6.2. Uncertainties in the data comparison

First of all, greenhouse conditions differ greatly from outdoor conditions, especially considering the dry and hot summer in which the previous experiment took place (Cazzaniga, 2020; unpublished). Research has shown that plant exudates differ under abiotic stresses, which is believed to an 'effort' of the plant to change to soil microbiome in way that the soil biota can help the plant with its stress (Hartman & Tringe, 2019; H. Liu et al., 2020; Timm et al., 2018). In the greenhouse conditions of this experiment, the soil was kept continuously wet and the greenhouse compartment was cooled by means of evaporative cooling on hot days, keeping abiotic stress to a minimum. Therefore, it may very well be that the differences measured between this research and the results by Obinu (2021; unpublished) are in part due to the changed plant exudates as a result of abiotic stress. On top of that, due to the fact that the plants were watered manually and the soil moisture was monitored manually as well, the possibility exists that one (group of) cover crop(s) received different amounts of water than others. Moreover, the crops could have taken up water at a different rate which could affect the soil moisture in between treatments. Due to these very reasons, the soil at the harvest time point showed quite some variance in its wetness, which showed to affect some of the taxa in this research, as mentioned in the results section. Moreover, since the soil wetness could be correlated to the type of cover crop grown (although no significant correlation was found), the effect of the cover crops could be in part due to differences in soil wetness. Moreover, earlier research suggests that soil moisture is a (strong) factor for the community composition in soils, which adds to the evidence that a difference in soil moisture could be problematic (Evans et al., 2014; W. Li et al., 2018; L. Ma et al., 2015).

Secondly, one cannot easily compare the relative abundance to an absolute abundance. Within this research, the relative abundance is defined as the abundance of a certain taxa given as a ratio of their respective phylum (usually next-generation sequencing results) and the absolute abundance is defined as any amount which is not presented in the form of a ratio (both counted number of organisms and qPCR data presented as an absolute value). As shown in figure 26 and 27, the total abundance of bacteria and fungi is affected by the cover crop treatments. If one was to measure the relative abundance, an absolute increase of a certain species could be missed since it would be masked by the increase of the respective phylum. E.g. if the abundance of a species increases by 10% and the phylum does so as well, the relative abundance would remain the same. Similarly, if one measures absolute abundance (like in this research) and finds an increase of a certain species, it might very well be that a large part of that increase is due to the favourable conditions of rhizosphere soil as opposed to bulk soil. In this case, attributing the measured effect solely to a species favouring one cover crop over the other would be faulty. Therefore, for any future research on this topic, a combination of absolute and relative abundance is recommended. In literature, no clear guideline on this topic was found as multiple papers were found within the field of soil antagonism that measured the absolute abundance (Adesina et al., 2007; Chen et al., 2017; Jaffuel et al., 2016), the relative abundance (Dong et al., 2016; Harkes et al., 2020; Qiu et al., 2012), or even both (Costa et al., 2006). However, it must be noted that the research objectives of these studies vary, as well as the fact that measuring an absolute number of antagonistic abundance in some of the mentioned papers relies on culturing the antagonists, which may not be possible for every antagonistic species. Moreover, if one wants to effectively choose between absolute and relative abundance, it should be investigated whether the functionality of the antagonists are dependent on their absolute or relative abundance in rhizosphere soil or that their antagonistic activity is not related to their (relative) abundance altogether.

Lastly, as mentioned in the materials & methods section of this paper, the soil that was used in this experiment came from four different groups (appendix 2). As the treatments were randomly distributed over the groups of soil, the influence of the different groups of soil should have been kept to a minimum. However, since it was found that a number of taxa (as mentioned in the results) were significantly affected by the batch of soil in which they grew, the batches of soil could have influenced the overall results one way or another. Similar to these results, numerous papers in literature can be found that show that the history of the soil affects the outcome of microbiome studies (Azarbad et al., 2020; Benitez et al., 2017; Kaplan et al., 2020; X. Li et al., 2019).

6.2.1. Contamination of *A. calcoaceticus*

As mentioned before, the qPCR's related to *A. calcoaceticus* were inconclusive. The primers used picked up something with a similar melt-curve and product length as *A. calcoaceticus*. After re-sterilizing the Milli-Q water, re-diluting the primers from stock, using new pipets, pipet-tips, tubes, and qPCR-plates, as well as putting in extra effort into working in a clean fashion, the contamination was still present. Moreover, the Cq values related to the negative control were all highly similar, making a contamination from bacteria present in the air and general environment unlikely. This leads us to believe that the contamination had to originate from the SYBR-Green IQ-mix or the primer stock. No record of *A. calcoaceticus* contamination originating from primer stock solutions or SYBR-Green IQ mixes have been found in literature.

6.2.2. *C. cladosporioides* unspecificity

As shown in figure 28, the product that was formed by using cc-3, a *C. cladosporioides* specific primer, did not approximate its theoretical value. Moreover, the product formed when primer pair cc-3 was run against a pure culture of *C. cladosporioides* was different from both the product formed by running this primer pair on any of the DNA samples or its theoretical value. Although no confidant explanation was found, it should be considered when validating the *C. cladosporioides* results.

7. Concluding remarks

The research presented here shows additional information on which cover crops can enhance the antagonists of *R. solani* and *P. penetrans,* as presented in the results section of this report. Moreover, the results presented here show that there is no indication that *R. solani* affects the abundance of the antagonists that were investigated in this paper. Moreover, when comparing this research to Cazzaniga (2020; unpublished); Obinu (2021; unpublished) it is shown that the abundance of some antagonist (e.g. Sphingomonadaceae) behave in a similar fashion in the rhizosphere of certain cover crops under different growing conditions while the abundance of others (e.g. Pseudomonadaceae) behave in a dissimilar fashion. Therefore, this research presents valuable information on which cover crop – antagonist interaction is more likely to be affected by contrasting growing conditions than others. Lastly, this research presents a ready-to-use protocol for primer development for relatively large groups of taxa in soil samples.

7.1. Further steps

In order to mitigate the uncertainty created by comparing absolute data with relative data, it is recommended that the primers developed in this research are run on the samples of Cazzaniga (2020; unpublished). For following research, the antagonistic of antagonists effect should be measured and correlated to the absolute and relative abundance of these antagonists to see which (absolute vs. relative abundance) is a better predicter for antagonism. However, it should be noted that any antagonistic effect produced by a predator-prey interaction might yield completely different results than an antagonistic effect produced by competition or any other type of interaction. Furthermore, LNA's should be added to the primer pair that require them for more trustworthy results. Moreover, the primer pairs that were only developed *in silico* should be ordered and tested *in vitro*.

All in all, although the data presented in this research may not be ready to use in an agricultural setting just yet, it is a small but sturdy step towards the holy grail of sustainable agriculture.

8. Appendix

Appendix 1. Selection of cover crops

Rizoctonia solani antagonists

Sphingobacteriaceae

As shown in *Figure 1,* the relative abundance of both DNA and cDNA of the Sphingobacteriaceae is significantly enhanced when in presence of the cover crop borage. The Sphingobacteriaceae family has shown to be antagonistic against *Rhizoctonia solani*, with an emphasis on *Pedobacter* (*sp.* V48 and *Pedobacter sp.* PR-M6) (Chapelle et al., 2016; de Boer et al., 2007; Garbeva et al., 2011; Song et al., 2017), which shows increased antagonistic effects in combination with *Pseudomonas fluorescens* (de Boer et al., 2007; Garbeva et al., 2011). This increased effect is attributed to an increased release of antimicrobial substances as a way to deal with the increased competition. For this very reason, the rhizosphere soil of borage in the pot experiment (as well as the corresponding controls) was checked for the abundance of *Pedobacter*.

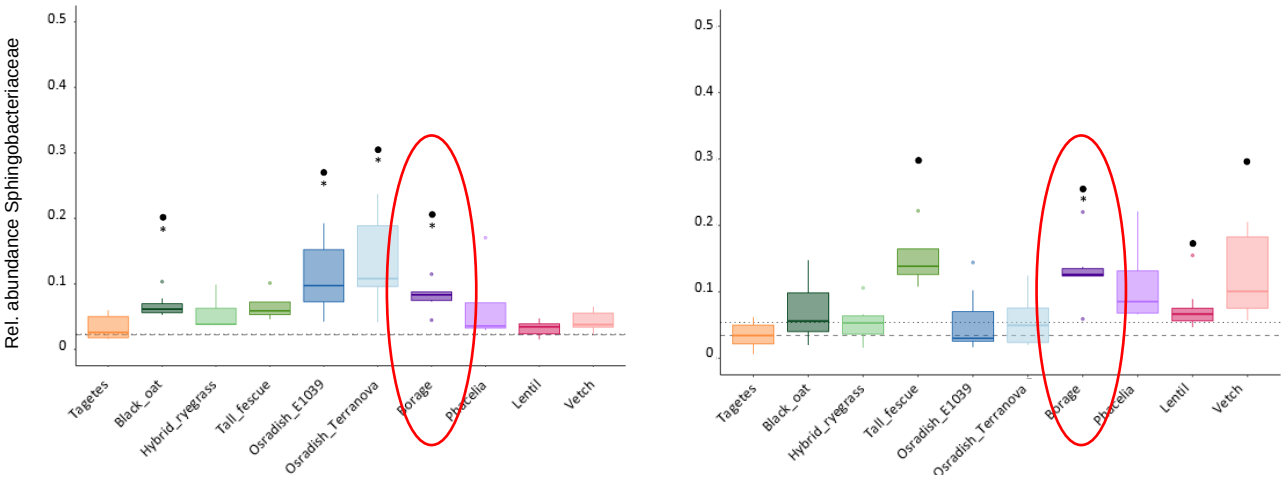


*Figure 24. The relative abundance of Sphingobacteriaceae as a result of common Dutch cover crops compared to a fallow control or to bulk soil. Significant differences compared to the fallow control are denoted by • , significant differences compared to bulk soil are denoted by * . Both the relative abundances of DNA (left) as well as cDNA (right) are shown. Figure provided by Obinu (2021; unpublished).*

Sphingomonadaceae

As shown in *Figure 2*, the relative abundance of the Sphingomonadaceae family is enhanced in the presence of black oat, for both DNA and cDNA. The Sphingomonadaceae have shown to be enhanced in rhizospheres of crops infected by *Rhizoctonia solani* (Chapelle et al., 2016; Mülner et al., 2019; C. Yin et al., 2013). Although the increased abundance of Sphingomonadaceae in the presence of *R. solani* has been attributed to its ability to decompose dead root material in the past (Wu et al., 2018), a dual culture assay has shown that the genus *Sphingomonas* show antagonistic traits (Chuntao Yin et al., 2021). Therefore, the rhizosphere soil of black oat in the pot experiment (as well as the corresponding controls) were checked for the abundance of *Sphingomonas.*

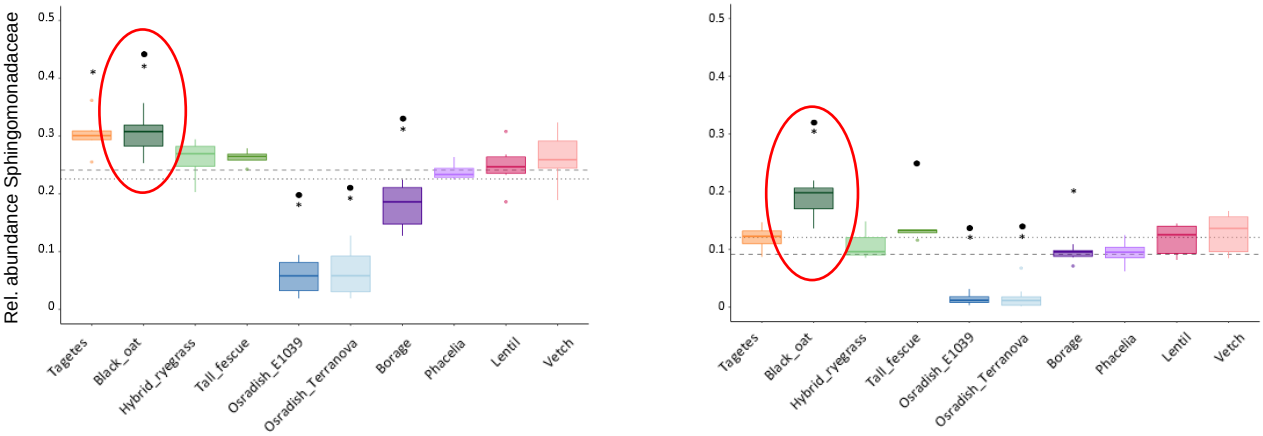


*Figure 25. The relative abundance of Sphingomonadaceae as a result of common Dutch cover crops. Significant differences compared to the fallow control are denoted by • , significant differences compared to bulk soil are denoted by * . Both the relative abundances of DNA (left) as well as cDNA (right) are shown. Figure provided by Obinu (2021; unpublished).*

Pseudomonadaceae

As shown in *Figure 3*, the relative abundance of the Pseudomonadaceae is enhanced in the presence of oilseed radish (osradish) and borage, for both DNA and cDNA. As stated before, *Pedobacter* shows a stronger antagonistic effect when in the presence of *Pseudomonas fluorescens,* belonging to the Pseudomonadaceae family. As both the Pseudomonadaceae and the Sphingobacteriaceae are enhanced by, among others, borage, the possibility remains that both *Pedobacter* and *Pseudomonas fluorescens* are enhanced by borage. *Pseudomonas fluorescens* has also been shown to have antagonistic traits against *R. solani* on its own, with this effect being attributed to either the production of antimicrobial substances, or competition for space on plant roots (Garbeva et al., 2011; Zachow et al., 2010). The rhizosphere soil of oilseed radish in the pot experiment will checked for the presence of *P. fluorescens.* Since borage is already being planted in the experiment, no additional pots will have to be used to check the rhizosphere soil of borage for the abundance of *P. fluorescens* as well. Moreover, *P. fluorescens* has shown to be an effective antagonist against *Pratylenchus penetrans* as well (Watson et al., 2018). Therefore, *P. fluorescens* will be checked for in the *P. penetrans* inoculated soils as well. *P. trivialis* has shown to be able to produce antifungal compounds as well (Kai et al., 2007) and as shown by Zachow et al. (2010), *P. trivialis* is especially effective in combination with *P. fluorescens*. Therefore, the rhizosphere of oilseed radish and borage were checked for *P. trivialis* as well.

Other Pseudomonadaceae species that have shown to have an antagonistic potential against *R. solani* include: *Pseudomonas* sp. E102, *Pseudomonas* sp. SaU7, and *Pseudomonas* sp. CMR12a (Garbeva et al., 2004; Z. Ma et al., 2016; Zachow et al., 2010).

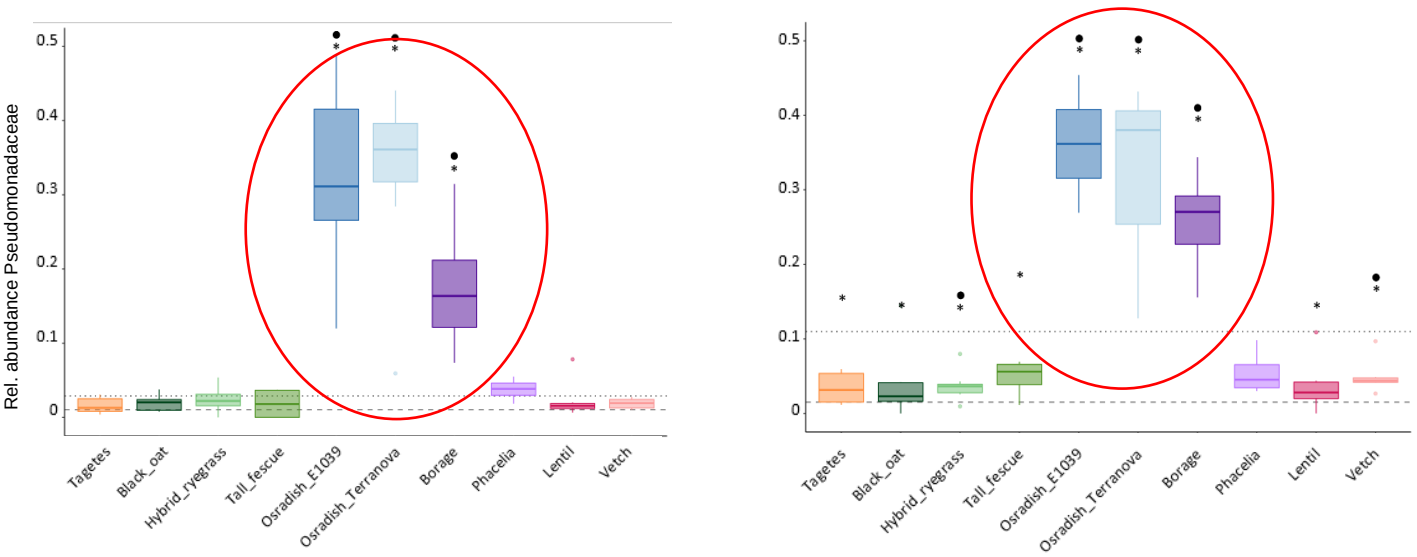


*Figure 26. The relative abundance of Pseudomonadaceae as a result of common Dutch cover crops compared to a fallow control or to bulk soil. Significant differences compared to the fallow control are denoted by • , significant differences compared to bulk soil are denoted by * . Both the relative abundances of DNA (left) as well as cDNA (right) are shown. Figure provided by Obinu (2021; unpublished).*

Pratylenchus penetrans antagonists

Acinetobacter

As shown in figure 4, the genus *Acinetobacter* is enhanced in terms of both DNA and cDNA when in the presence of Oilseed radish. Within the genus *Acinetobacter, A. calcoaceticus* has shown to have antagonistic potential against fungi (Zhao et al., 2018), among which *R. solani* (Donmez et al., 2015). Similar to *A. calcoaceticus*, *A. baumannii* shows antagonistic traits against *R. solani* (C. H. Liu et al., 2007). However, due to the extreme similarities between *A. calcoaceticus* and *A. baumanni* (Gerner-Smidt, 1992), *A. baumanni* was not tested for.

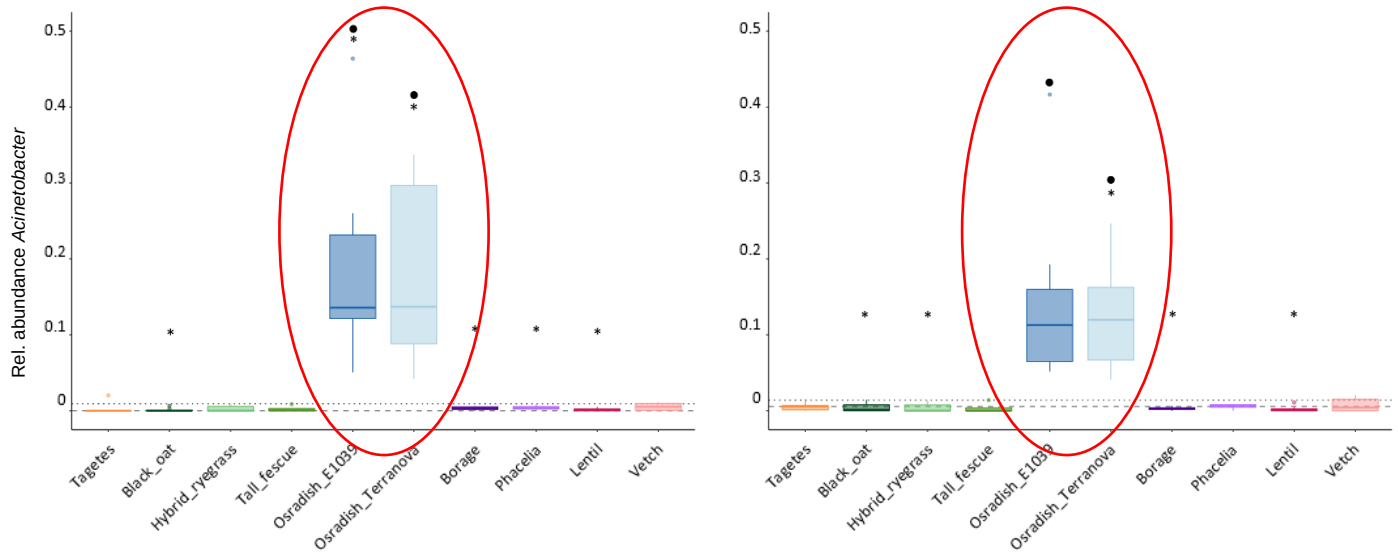


*Figure 27. The relative abundance of Acinetobacter as a result of common Dutch cover crops compared to a fallow control or to bulk soil. Significant differences compared to the fallow control are denoted by • , significant differences compared to bulk soil are denoted by * . Both the relative abundances of DNA (left) as well as cDNA (right) are shown. Figure provided by Obinu (2021; unpublished).*

Aspergillaceae

As shown in *figure 5*, the family Aspergillaceae family is significantly enhanced when in the presence of lentil for both DNA and cDNA. Shemshura et al. (2016) showed that *Aspergillus candidus* has an antagonistic effect on nematodes because of metabolites that are specific to *Aspergillus*. Other studies find similar results stating that *A. candidus* has an anti-nematode/microbial effect due to specific metabolites (Elaasser et al., 2011; Qureshi et al., 2012). Although the antagonistic compound is not specific to *A. candidus* other species like *A. flavus, A. oryzae* and *A. niger* have been described in literature as phytopathogenic (El-Baky et al., 2021; Manwar et al., 2004), which makes them less than ideal candidates for enhancement by cover crops. No record of pathogenic infection of live plant material by *A. candidus* has been found. Therefore, only the presence of *A. candidus* was investigated.

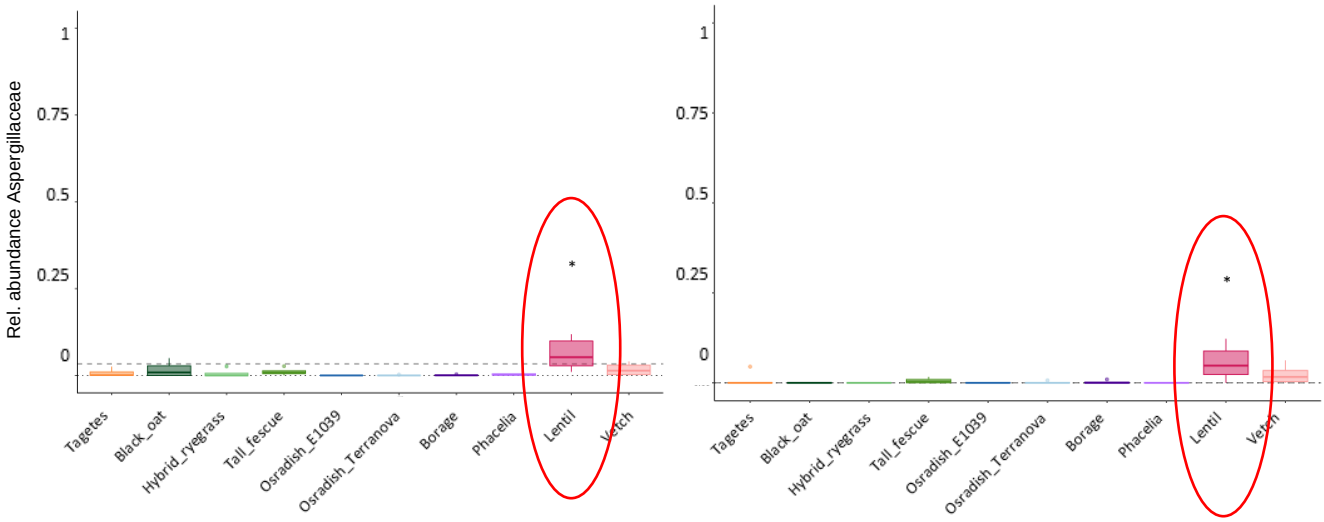


*Figure 28. The relative abundance of Aspergillaceae as a result of common Dutch cover crops compared to a fallow control or to bulk soil. Significant differences compared to the fallow control are denoted by • , significant differences compared to bulk soil are denoted by * . Both the relative abundances of DNA (left) as well as cDNA (right) are shown. Figure provided by Obinu (2021; unpublished).*

Cladosporiaceae

As shown in figure 6, the Cladosporiaceae family is significantly enhanced in the presences of multiple cover crops. In this research, phacelia will be chosen as cover crop to be able to highlight and investigate the apparent differences between borage and phacelia, both members of Boraginaceae. For instance, Meyer et al. (2004) showed that *Cladosporium cladosporioides* could inhibit the hatching of nematodes *Meloidogyne incognita* and *Heterodera glycines* although results were inconsistent. Similar results were obtained by Sun et al. (2006) for *M. incognita*. It should be noted that *C. cladosporioides* is known as a fungal pathogen in strawberries and grapes (Mengal et al., 2020; Nam et al., 2015).

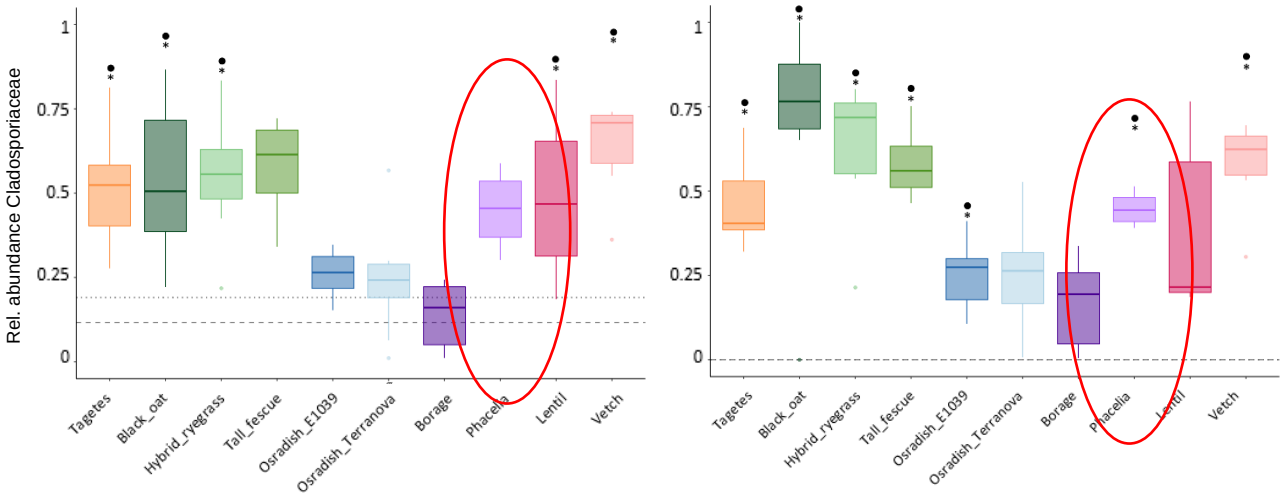


*Figure 29. The relative abundance of Cladosporiaceae as a result of common Dutch cover crops compared to a fallow control or to bulk soil. Significant differences compared to the fallow control are denoted by • , significant differences compared to bulk soil are denoted by * . Both the relative abundances of DNA (left) as well as cDNA (right) are shown. Figure provided by Obinu (2021; unpublished).*

Appendix 2. schematic overview pot experiment

A schematic overview of the pot experiment setup with corresponding legend

76 BO, NP	71 BO, RS	66 L, PP	61 O, PP	56 O, NP	51 NC, RS	46 L, PP	41 NC, RS	36 NC, NP	31 NC, PP	26 P, NP	21 NC, PP	16 BO, RS	11 O, NP	6 BO, NP	1 L, NP
77 NC, PP	72 O, RS	67 NC, NP	62 NC, NP	57 O, NP	52 NC, PP	47 BO, RS	42 P, PP	37 BO, NP	32 NC, NP	27 BO, NP	22 B, RS	17 P, NP	12 L, PP	7 NC, NP	2 P, PP
78 O, NP	73 P, PP	68 P, NP	63 O, PP	58 P, PP	53 O, RS	48 O, RS	43 O, NP	38 B, RS	33 O, NP	28 B, RS	23 NC, RS	18 P, NP	13 NC, NP	8 L, PP	3 O, RS
79 NC, NP	74 B, NP	69 L, NP	64 NC, NP	59 NC, RS	54 BO, NP	49 L, NP	44 L, NP	39 B, NP	34 BO, RS	29 O, PP	24 L, PP	19 L, NP	14 O, PP	9 O, PP	4 P, PP
80 B, RS	75 NC, RS	70 O, NP	65 NC, NP	60 O, NP	55 P, NP	50 B, NP	45 B, RS	40 BO, RS	35 NC, NP	30 O, RS	25 B, NP	20 B, NP	15 O, NP	10 O, NP	5 NC, PP

barrel #1		Borage = B		R. solani = RS	
barrel #2		Black oat = BO		P. penetrans = PP	
barrel #3		Oilseed radish = O		no pathogen = NP	
barrel #4		Lentil = L			
		Phacelia = P			
		No cover crop = NC			

Appendix 3. Primer selection

Below a selection of the primers can be found along with a legend explaining the table. Please note that not all displayed primers were ordered for *in vitro* testing, as indicated by the column 'ordered'.

legend	
acine	Acinetobacter
acal	Acinetobacter calcoaceticus
acan	Aspergillus candidus
asp	Aspergilliaceae
bac	Universal bacterial primer
c	Cladosporiaceae
cc	Cladosporium cladosporioides
p	Pseudomonadaceae
pedo	Pedobacter
pf	Pseudomonas fluorescens
pt	Pseudomonas trivialis
sbc	Sphingobacteriaceae
sm	Sphingomonas
smc	Sphingomonadaceae
fw	forward primer
rv	reverse primer
()	LNA nucleotide
[A/t]	Degenerate nucleotide (example: predominantly A, also contains T)
	primer found in literature

Primer	sequence	Ordered	Tm	contains LNA?	# troublesome unindended targets	# total unintended targets	notes
acal-fw1	GACGATCTGTAGCGGGTCTG	✓	64.5		0	3	
acal-rv1	GTAAGTTCCCGAAGGCACCA	✓	65.1		0	3	
acal-fw2	CCACACTGGGACTGAGACAC	✓	64.7		0	3	
acal-rv2	TGTAAGTTCCCGAAGGCACC	✓	65.1		0	3	
acal-fw3	ACGCGAAGAACCTTACCTGG	✓	65		0	3	
acal-rv3	CCCAACATCTCACGACACGA	✓	64.9		0	3	
acal-fw4	GGTGCCTTCGGGAACTTACA		65.1		0	3	
acal-rv4	AGGGCCATGATGACTTGACG		65.2		0	3	
acal-fw5	GACAACAGTTATAAGGTTTCAGGTG		62.8		1		Higgins et al. (2020), 1 mismatch with intended target
acal-rv5	CCGCTATCTGTATCCGCAGTA		64		1		Higgins et al. (2020), 1 mismatch with intended target
acine-fw1	GCCAGTGACAAACTGGAGGA	✓	65.1		0	0	
acine-rv1	CAAACTCCCATGGTGTGACG	✓	64		0	0	
acine-fw2	TCAGAATGCCGCGGTGAATA	✓	65.1		0	0	
acine-rv2	CTTCTGGTGCAACAAACTCCC	✓	64.5		0	0	
acine-fw3	ACGCGAAGAACCTTACCTGG	✓	65		0	0	
acine-rv3	AAACTCCCATGGTGTGACGG	✓	65.4		0	0	
acine-fw4	TTCGATGCAACGCGAAGAAC		64.6		0	0	
acine-rv4	TCTGGTGCAACAAACTCCCAT		65.6		0	0	
acine-fw5	GGCGACGATCTGTAGCGGGTCTG		70.2		3		Xin et al. (2014), no acinetobacter found as target
acine-rv5	TGGGAGAGGATGGTAGAATTCCAG		65.9		3		Xin et al. (2014), no acinetobacter found as target
sm-fw1	GGTGATAAGCCGGAGGAAGG	✓	64.8		0	1	
sm-rv1	GCC(T)GGGAACGTATTCACCG	✓	65.7	✓	0	1	requires LNA
sm-fw2	CTGGTAGTCCACGCCGTAAA	✓	64.7		0	1	
sm-rv2	equals sm-rv1		65.7	(✓)	0	1	
sm-fw3	GATACCCTGGTAGTCCACGC	✓	64.4		0	1	
sm-rv3	equals sm-rv1		65.7	(✓)	0	1	
sm-fw4	CGTGGGGAGCAAACAGGATT		65.8		0	1	
sm-rv4	equals sm-rv1		65.7		0	1	
sm-fw5	ACGATGATAACTAGCTG		54		a lot		Kim et al. (1998)
sm-rv5	CTCTCGAGTTGCAGAGA		58.7		a lot		Kim et al. (1998)
smc-fw1	CCACACTGGGACTGAGACAC	✓	64.7		0	10	
smc-rv1	GTTTACGGCGTGGACTACCA	✓	64.9		0	10	
smc-fw2	TAATTCGAAGCAACGCGCAG	✓	64.7		0	10	
smc-rv2	CCCAACATCTCACGACACGA	✓	64.9		0	10	
smc-fw3	TCGTGTCGTGAGATGTTGGG	✓	64.9		0	10	
smc-rv3	CCTTCCTCCGGCTTATCACC	✓	64.8		0	10	
smc-fw4	TGACGTCAAGTCCTCATGGC		65		0	10	
smc-rv4	GCATGCTGATCCGCGATTAC		64.4		0	10	
smc-fw5	AGAGTTTGATC[M]TGGCTCAG		61.6		a lot		Boersma et al. (2020)
smc-rv5	GGTTACCTTGTTACGACTT		58.2		a lot		Boersma et al. (2020)
cc-fw1	GGTCTTCTGTCCCCTAAGCG	✓	64.6		0	0	
cc-rv1	GTTGTTTTACGGCGTAGCCTC	✓	64.4		0	0	
cc-fw2	TCCGGGTGGACACTTCAAAC	✓	65.3		0	0	
cc-rv2	GTTGTTTTACGGCGTAGCCT	✓	63.9		0	0	
cc-fw3	TGACCCCGGTCTAACCACC	✓	66.2		0	0	
cc-rv3	GTTGCCCAATACCAAGCGAG	✓	64.4		0	0	
cc-fw4	AAGCCTCGCTTGGTATTGGG		65.6		0	0	
cc-rv4	GGTTGTTTTACGGCGTAGCCT		65.9		0	0	
c-fw1	CAAGCCTCGCTTGGTATTGG	✓	64.2		0	1	
c-rv1	TTAGCGAATAGTTTCCACAACGC	✓	64.5		0	1	
c-fw2	AGCCTCGCTTGGTATTGGG	✓	65.2		0	1	
c-rv2	CTTTAGCGAATAGTTTCCACAACGC	✓	65.3		0	1	
c-fw3	ACGGATCTCTTGGTTCTGGC	✓	64.9		0	0	
c-rv3	ACCAAGC[G]A[G]GCTTGAGTG	✓	65.5	✓	0	0	LNA, unintended targets that are 'filtered out' by LNA have not yet been checked for possibility of occurrence in soil and are thus not listed in this table

							deviation of about 5%, degenerate could be possible but might not be necessary, specificity with degenerate has not yet been verified
c-fw4	GTTCGAGCGTCATT[T/a]CACCAC		64.6		0	3	
c-rv4	CCCAATACCAAGCGAGGCTT		65.6		0	3	
pt-fw1	AGTACGGCCGCAAGGTTAAA	✓	65.3		2/4 depending on LNA	3/9 depending on LNA	
pt-rv1	AGAGTGCCCACCAT(T)ACGTG	✓	65.1	✓	2/4 depending on LNA	3/9 depending on LNA	Requires LNA
pt-fw2	CACGT(A)ATGGTGGGCACTCT	✓	65.1	✓	2/4 depending on LNA	3/9 depending on LNA	Requires LNA
pt-rv2	AGCTACTTCTGGTGCAACCC	✓	65.2		2/4 depending on LNA	3/9 depending on LNA	
pt-fw3	TAGCCGTTGGAAGCCTTGAG	✓	65.1		4	11	
pt-rv3	TTTAACCTTGCGGCCGTACT	✓	65.3		4	11	
pt-fw4	ATTAAGTTGACCGCCTGGGG		65.3		4	9	
pt-rv4	CAGACTGCGATCCGGACTAC		64.5		4	9	
pf-fw1	GTAAAGCCTAGGAATCTGCC		61		a lot		philippes file
pf-rv1	AACCCGAAGACCTTCTT(A)AC		60.7		a lot		philippes file, no 100% match with downloaded pseudomonas fluorescens sequence
pf-fw2	GTCGAGCGGTAGAGAGAAGC	✓	64.4		0/3 depending on LNA	0/6 depending on LNA	might not pick up all pf
pf-rv2	GACCTTCTT(A)ACACACGCGG	✓	63.9	✓	0/3 depending on LNA	0/6 depending on LNA	might not pick up all pf
pf-fw3	CCGCGTGTGT(T)AAGAAGGTC	✓	63.9	✓	0/3 depending on LNA	0/6 depending on LNA	might not pick up all pf
pf-rv3	CCCGGGGATTTCACATCCAA	✓	65.4		0/3 depending on LNA	0/6 depending on LNA	might not pick up all pf
pf-fw4	ATTAAGTTGACCGCCTGGGG	✓	65.3		5	6	will pick up all pf
pf-rv4	AGCTACTTCTGGTGCAACCC	✓	65.2		5	6	will pick up all pf
pf-fw5	TCTAAGGAGACTGCCGGTGA	✓	65.3		5	6	will pick up all pf
pf-rv5	ACTTCTGGTGCAACCCACTC	✓	65.4		5	6	will pick up all pf
p-fw1	CTGGAACTGAGACACGGTCC	✓	64.7		2	2	
p-rv1	CCCAACATCTCACGACACGA	✓	64.9		2	2	
p-fw2	TCGTGTCGTGAGATGTTGGG	✓	64.9		2	2	
p-rv2	TCACCGGCAGTCTCCTTAGA	✓	65.3		2	2	
p-fw3	CAGCTCGTGTCGTGAGATGT	✓	64.9		2	2	
p-rv3	ACCGGCAGTCTCCTTAGAGT	✓	65.3		2	2	
p-fw4	GCAACGCGAAGAACCTTACC		64.5		2	2	
p-rv4	AGGGCCATGATGACTTGACG		65.2		2	2	
							Has one unintended target when ran against the 'nr' database of NCBI
bac-fw1	CGCGATAGCGAGCTTCCGA	✓	67		0	0	
bac-rv1	CTGCTGCCTCCCGTAGGAGT	✓	68.2		0	0	
bac-fw2	CACGCGATAGCGAGCTTCC	✓	65.7		1	1	
bac-rv2	GCTGCCTCCCGTAGGAGT	✓	66.2		1	1	
pedo-fw1	AAACTAGGTGCTGCATGGCT		65.5		2		
pedo-rv1	CTTGACGGGCGGTGTGT		64.7		2		
pedo-fw2	AGACTGCCTGTGCAAACAGA		65.3		2		
pedo-rv2	ATGGCTTGACGGGCGG		65.7		2		
pedo-fw3	AGTCCACGCCCTAAACGATG		65.1		2		
pedo-rv3	GCTTGACGGGCGGTGT		65.8		2		
pedo-fw4	CCTTACCCGGGCTTGAAAGT		65.2		2		
pedo-rv4	CTTCCATGGCTTGACGGGC		66.3		2		might not be specific to all pedobacter, could maybe be solved by a degenerate but specificity with degenerate has not yet been verified

sbc-fw1	TAACTCCGTGCCAGCAGC	65.4	0	
sbc-rv1	AGGTTCCTCGCGTATCATCG	64.5	0	
sbc-fw2	TCGATGATACGCGAGGAACC	64.5	0	
sbc-rv2	CGAGCTGACGACAGCCAT	65	0	
sbc-fw3	CUAACUCCGUGCCAGCAGC	65.8	0	
sbc-rv3	GGTTCCTCGCGTATCATCGAA	64.9	0	
sbc-fw4	GCTAACTCCGTGCCAGCAG	65.8	0	
sbc-rv4	AAGGTTCCTCGCGTATCATCG	64.9	0	
acan-fw1	CCACCCGTGTATACCGTACC	64.3	1	
acan-rv1	ATCGATGCCGGAACCAAGAG	65.2	1	
acan-fw2	ACCCGTGTATACCGTACCCT	65.1	2	
acan-rv2	AGACAGTGTTCGTGTTGGGG	65.2	2	
acan-fw3	CACCCGTGTATACCGTACCC	64.3	2	
acan-rv3	CGATGCCGGAACCAAGAGAT	65.2	2	
acan-fw4	ACGCAGCGAAATGCGATAAC	64.8	2	
acan-rv4	TGACAAAGCCCCATACGCTC	65.4	2	
asp-fw1	ATCTCTTGGTTCCGGCATCG	65.2	0	all on very similar place on genome
asp-rv1	GCGCAATGTGCGTTCAAAGA	65.5	0	all on very similar place on genome
asp-fw2	ATCGATGAAGAACGCAGCGA	65.2	0	all on very similar place on genome
asp-rv2	CGCAATGTGCGTTCAAAGAC	63.7	0	all on very similar place on genome
asp-fw3	ATCGATGAAGAACGCAGCGAA	65.6	0	all on very similar place on genome
asp-rv3	TGTGCGTTCAAAGACTCGATG	64	0	all on very similar place on genome
asp-fw4	TCCGGCATCGATGAAGAACG	65.3	0	all on very similar place on genome
asp-rv4	GGCGCAATGTGCGTTCAA	65.3	0	all on very similar place on genome

Appendix 4. Unintended targets

Described below are the unintended that are picked up by the primers as indicated by 'NCBI primer BLAST' in alphabetical order of the primer pairs.

Primer pair:	acal-fw1 acal-rv1		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Acinetobacter seifertii	non-troublesome	Human microbiome	Nemec et al. (2015)
Acinetobacter lactucae	unlikely	Post-harvest iceberg lettuce	Rooney et al. (2016)
Acinetobacter dispersus	non-troublesome	Well water	Nemec et al. (2016)

Primer pair:	acal-fw2 acal-rv2		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Acinetobacter seifertii	non-troublesome	Human microbiome	Nemec et al. (2015)
Acinetobacter lactucae	unlikely	Post-harvest iceberg lettuce	Rooney et al. (2016)
Acinetobacter dispersus	non-troublesome	Well water	Nemec et al. (2016)

Primer pair:	acal-fw3 acal-rv3		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Acinetobacter seifertii	non-troublesome	Human microbiome	Nemec et al. (2015)
Acinetobacter lactucae	unlikely	Post-harvest iceberg lettuce	Rooney et al. (2016)
Acinetobacter dispersus	non-troublesome	Well water	Nemec et al. (2016)

Primer pair:	acine-fw1 acine-rv1		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
none			

Primer pair:	acine-fw2 acine-rv2		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
none			

Primer pair:	acine-fw3 acine-rv3		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
none			

Primer pair:	bac-fw1 bac-rv1		
Unintended target: none	**Marked as:**	**Isolated from / habitat:**	**Reference:**

Primer pair:	bac-fw2 bac-rv2		
Unintended target: Pleurotus ostreatus	**Marked as:** Troublesome	**Isolated from / habitat:** Diverse soils	**Reference:** Sánchez (2010)

Primer pair:	c-fw1 c-rv1		
Unintended target: Metarhizium album	**Marked as:** Non-troublesome	**Isolated from / habitat:** Leaf- and plant hoppers	**Reference:** Rombach et al. (1987)

Primer pair:	c-fw2 c-rv2		
Unintended target: Metarhizium album	**Marked as:** Non-troublesome	**Isolated from / habitat:** Leaf- and plant hoppers	**Reference:** Rombach et al. (1987)

Primer pair:	c-fw2 c-rv2		
Unintended target: Metarhizium album	**Marked as:** Non-troublesome	**Isolated from / habitat:** Leaf- and plant hoppers	**Reference:** Rombach et al. (1987)

Primer pair:	c-fw3 c-rv3	Requires LNA in order to have zero unintended targets	
Unintended target: None	**Marked as:**	**Isolated from / habitat:**	**Reference:**

Primer pair:	cc-fw1 cc-rv1		
Unintended target: None	**Marked as:**	**Isolated from / habitat:**	**Reference:**

Primer pair:	cc-fw2 cc-rv2		
Unintended target: None	**Marked as:**	**Isolated from / habitat:**	**Reference:**

Primer pair:	cc-fw3 cc-rv3		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
None			

Primer pair:	p-fw1 p-rv1		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Rhodanobacter panaciterrae	Troublesome	Ginseng field soil	Wang et al. (2011)
Dyella flava	Troublesome	Forest soil	Xia et al. (2017)

Primer pair:	p-fw2 p-rv2		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Rhodanobacter panaciterrae	Troublesome	Ginseng field soil	Wang et al. (2011)
Dyella flava	Troublesome	Forest soil	Xia et al. (2017)

Primer pair:	p-fw3 p-rv3		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Rhodanobacter panaciterrae	Troublesome	Ginseng field soil	Wang et al. (2011)
Dyella flava	Troublesome	Forest soil	Xia et al. (2017)

Primer pair:	pf-fw2 pf-rv2	Possibility of including an LNA exists	
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pseudomonas lactucae	Troublesome	Pre-harvest lettuce	Sawada et al. (2021)
Pseudomonas salomonii	Troublesome	Garlic plants	Gardan et al. (2002)
Pseudomonas orientalis	Unlikely	Foods	Leja et al. (2019)
Pseudomonas pisciculturae	Non-troublesome	Rainbow trout	Duman et al. (2021)
Pseudomonas chlororaphis	Troublesome	Rhizosphere, shows antagonistic potential against *P. penetrans*	Hackenberg et al. (2000); J. Li et al. (2015)
Pseudomonas paracarnis	Non-troublesome	Refrigerated beef	Lick et al. (2021)

Primer pair:	pf-fw3 pf-rv3	Possibility of including an LNA exists	
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pseudomonas lactucae	Troublesome	Pre-harvest lettuce	Sawada et al. (2021)
Pseudomonas salomonii	Troublesome	Garlic plants	(Gardan et al., 2002)
Pseudomonas orientalis	Unlikely	Foods	Leja et al. (2019)
Pseudomonas pisciculturae	Non-troublesome	Rainbow trout	(2021)
Pseudomonas chlororaphis	Troublesome	Rhizosphere, shows antagonistic potential against *P. penetrans*	Hackenberg et al. (2000); J. Li et al. (2015)
Pseudomonas paracarnis	Non-troublesome	Refrigerated beef	Lick et al. (2021)

Primer pair:	pf-fw4 pf-rv4		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pseudomonas donghuensis	Troublesome	Agricultural soil, shows antagonistic effects	Marin-Bruzos et al. (2021); Muzio et al. (2020)
Pseudomonas palmensis	Troublesome	Rhizosphere of mediterranean shrub	Gutierrez-Albanchez et al. (2021)
Pseudomonas atacamensis	Troublesome	Rhizosphere of dessert plants	Elmahi et al. (2021); Poblete-Morales et al. (2020)
Pseudomonas glycinae	Troublesome	Soybean rhizosphere	Jia et al. (2020)
Pseudomonas foliumensis	Troublesome	Wheat leaf necrosions	Tambong et al. (2021)
Pseudomonas salina	Non-troublesome	Salt water lakes	Zhong et al. (2015)

Primer pair:	pf-fw5 pf-rv5		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pseudomonas donghuensis	Troublesome	Agricultural soil, shows antagonistic effects	Marin-Bruzos et al. (2021); Muzio et al. (2020)
Pseudomonas palmensis	Troublesome	Rhizosphere of mediterranean shrub (Nicotiana Glauca)	Gutierrez-Albanchez et al. (2021)
Pseudomonas atacamensis	Troublesome	Rhizosphere of dessert plants	Elmahi et al. (2021); Poblete-Morales et al. (2020)
Pseudomonas glycinae	Troublesome	Soybean rhizosphere	Jia et al. (2020)
Pseudomonas foliumensis	Troublesome	Wheat leaf necrosions	Tambong et al. (2021)
Pseudomonas salina	Non-troublesome	Salt water lakes	Zhong et al. (2015)

Primer pair:	pt-fw1 pt-rv1	Possibility of including an LNA exists	
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pseudomonas edaphica	Troublesome	Rhizosphere of Mediterranean shrub (Cistus ladanifer L.)	Ramírez-Bahena et al. (2019)
Pseudomonas kairouanensis	Unlikely	Citrus plants' leaf	Oueslati et al. (2019)
Pseudomonas lurida	Troublesome	Among others; grass phyllosphere	Behrendt et al. (2007); Selvakumar et al. (2011)
Pseudomonas cremoris	Non-troublesome	Processed dairy products	Hofmann et al. (2021)
Pseudomonas salomonii	Troublesome	Garlic plants	Gardan et al. (2002)
Pseudomonas reactans	Non-troublesome	Cockroaches	Zhang et al. (2013)
Pseudomonas nabeulensis	Unlikely	Citrus plants' leaf	Oueslati et al. (2019)
Pseudomonas tolaasii	Troublesome	Mushrooms	Nair and Fahy (1972)
Pseudomonas palleroniana	Troublesome	Rice seeds	Gardan et al. (2002)

Primer pair:	pt-fw2 pt-rv2	Possibility of including an LNA exists	
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pseudomonas edaphica	Troublesome	Rhizosphere of Mediterranean shrub (Cistus ladanifer L.)	Ramírez-Bahena et al. (2019)
Pseudomonas kairouanensis	Unlikely	Citrus plants' leaf	Oueslati et al. (2019)
Pseudomonas lurida	Troublesome	Among others; grass phyllosphere	Behrendt et al. (2007); Selvakumar et al. (2011)
Pseudomonas cremoris	Non-troublesome	Processed dairy products	Hofmann et al. (2021)
Pseudomonas salomonii	Troublesome	Garlic plants	Gardan et al. (2002)
Pseudomonas reactans	Non-troublesome	Cockroaches	Zhang et al. (2013)
Pseudomonas nabeulensis	Unlikely	Citrus plants' leaf	Oueslati et al. (2019)
Pseudomonas tolaasii	Troublesome	Mushrooms	Nair et al. (1972)
Pseudomonas palleroniana	Troublesome	Rice seeds	Gardan et al. (2002)

Primer pair:	pt-fw3 pt-rv3		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pseudomonas pisciculturae	Non-troublesome	Rainbow trout	Duman et al. (2021)
Pseudomonas cremoris	Non-troublesome	Processed dairy products	Hofmann et al. (2021)
Pseudomonas edaphica	Troublesome	Rhizosphere of a Mediterranean shrub (Cistus ladanifer L.)	Ramírez-Bahena et al. (2019)
Pseudomonas salomonii	Troublesome	Garlic plants	Gardan et al. (2002)
Pseudomonas reactans	Non-troublesome	Cockroaches	Zhang et al. (2013)
Pseudomonas nabeulensis	Unlikely	Citrus plants' leaf	Oueslati et al. (2019)
Pseudomonas kairouanensis	Unlikely	Citrus plants' leaf	Oueslati et al. (2019)
Fredinandcohnia aciditolerans	Troublesome	Paddy soil	Ding et al. (2019)
Pseudomonas tolaasii	Troublesome	Mushrooms	Nair et al. (1972)
Pseudomonas lurida	Troublesome	Among others; grass phyllosphere	Behrendt et al. (2007); Selvakumar et al. (2011)
Pseudomonas palleroniana	Troublesome	Rice seeds	Gardan et al. (2002)

Primer pair:	bac-fw1 bac-rv1		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
None			

Primer pair:	bac-fw2 bac-rv2		
Unintended target:	**Marked as:**	**Isolated from / habitat:**	**Reference:**
Pleurotus ostreatus	Troublesome	Temperate forest soils	Sánchez (2010); Vilgalys et al. (1993)

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