Impact of Brassicaceae Cover Crops on Pea Root Rot (*Aphanomyces euteiches*) in Subsequent Peas

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Abstract

The soil-borne pathogen *Aphanomyces euteiches* Drechs is among the greatest threats to pea production world-wide. This pathogen can persist in soil for many years without a host plant and is very difficult to control due to its long-lived oospores and to environmental restrictions on fungicide application. Brassicaceae (brassica) cover crops, which are already used in agricultural cropping systems to reduce nutrient leaching and prevent soil erosion, can also be used to control some soil-borne pathogens. Most brassica plants contain glucosinolates (GSLs) and some hydrolysis products of GSLs, such as isothiocyanates (ITCs), can be highly toxic to soil organisms. Different vegetative parts of the plant and different brassica species contain different GSLs.

In his thesis, two brassica species with different GSL profiles, *Brassica juncea* and *Sinapis alba*, and the pathogen *A. euteiches* were used as models in bio-fumigation studies. *In vitro* experiments showed that volatiles produced from *B. juncea* shoot tissue strongly inhibited growth of *A. euteiches* mycelium, while volatiles from *S. alba* tissue had a weaker effect. However, a direct bioassay following incorporation of fresh brassica tissues into *A. euteiches*-infected soil showed no suppression of root rot in pea plants.

Further *in vitro* experiments showed that ITC concentration and duration of exposure were both essential factors for the inhibitory effect on the pathogen. Analysis of the initial ITC production from the two brassica species used as models showed different release patterns, with *S. alba* tissue showing more immediate production of ITCs than *B. juncea* tissue.

There was significant suppression of pea root rot when *S. alba* was grown for 11 weeks in an *A. euteiches*-infected soil. ITCs were detected in the rhizosphere of the growing brassica crops and the variety of ITCs produced by *S. alba* roots appeared to be the main factor for inhibition. Problems can arise in bio-fumigation if beneficial organisms such as N_2-fixing bacteria and ammonia-oxidising organisms are also suppressed. However, real-time PCR analysis to quantify gene copies of key enzymes involved in ammonia oxidation and N_2 fixation in soil sampled after 10 weeks of cover crop growth showed that the N_2-fixing bacterial communities and the ammonia-oxidising bacteria and archaea were not negatively affected by the growing brassicas.

When using brassicas to control soil-borne pathogens, important factors are the choice of brassica species, a sufficient concentration of volatile ITCs and exposure for a sufficient period of time, which may require soil covering after brassica tissue incorporation.

Keywords: glucosinolates, isothiocyanates, nutrients, N_2-fixing bacteria, nitrifying community

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Dedication

In memory of my father

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Hossain, S.**, Bergkvist, G., Berglund, K., Mårtensson, A. and Persson, P. 2012. *Aphanomyces* pea root rot disease and control with special reference to impact of Brassicaceae cover crops. *Acta Agriculturae Scandinavica, Section B - Soil & Plant Science* 62(6):477-487.

II **Hossain, S.**, Bergkvist, G., Berglund, K., Mårtensson, A., Glinwood, R. and Persson, P. Growth of *Sinapis alba* in *Aphanomyces euteiches* infested soil reduces pea root rot in subsequent peas (manuscript).

III **Hossain, S.**, Bergkvist, G., Berglund, K., Mårtensson, A. Hallin, S. and Persson, P. Influence of Brassicaceae cover crops on ammonia-oxidising and nitrogen-fixing organism communities (manuscript).

IV **Hossain, S.**, Bergkvist, G., Berglund, K., Mårtensson, A., Glinwood, R., Kabouw, P. and Persson, P. Concentration and time-dependent effects of isothiocyanates produced from Brassicaceae shoot tissues on the pea root rot pathogen *Aphanomyces euteiches* (manuscript).

1 Introduction

The research presented in this thesis arose from Swedish farmers' interest in using Brassicaceae cover crops as sanitisers of soil-borne pathogens and plant parasitic nematodes. However, knowledge of how the crops should be used or managed, which crop species to be used and what effects can be expected is limited. Therefore different brassica plants, the soil-borne pathogen *Aphanomyces euteiches* causing pea root rot and pea plants were used as models to investigate the system and possible mechanisms behind interactive effects.

Field pea (*Pisum sativum* L.) is a valuable crop globally due to its high protein content (up to 26%) and its high concentrations of the essential amino acids lysine and tryptophan, its atmospheric N_2-fixing ability and its single or mixed growing habit (Elzebroek and Wind, 2008). Field peas contain high levels of carbohydrates and 86-87% of total nutrients are digestible. The global acreage of dry peas increased from 1980 to 1990 and then gradually declined, but the overall yield has continued to increase (FAO, 2013). However, cultivation of peas is difficult due to soil compaction, low pH and the soil-borne pathogen *Aphanomyces euteiches* Drechs causing pea root rot, a serious disease which is very difficult to control.

The *A. euteiches* pathogen is globally distributed and can cause disease in many legume plants, but peas show the greatest economic losses (Papavizas and Ayers, 1974; Gaulin et al., 2007). The pathogen can survive in the soil for a long time without a host plant, due to the thick protective cell walls of its oospores (Pfender and Hagedorn, 1983). Previous surveys in Sweden and Denmark showed that 42% and 38%, respectively, of soil samples were contaminated with *A. euteiches* (Persson et al., 1997). The disease greatly hampers pea production in these countries, particularly on clayey soils in humid areas (Persson, 2008). Pea root rot is difficult to control as there are no available pea cultivars with an acceptable level of resistance (McGee et al., 2012). In addition, fungicides are prohibited as soil treatments in many

countries. For these reasons, peas are not recommended for re-cultivation in *A. euteiches*-contaminated soil for many years. There is a great need for a sustainable solution to the threat of *A. euteiches* for safe pea production in future agriculture.

There has long been interest in Brassicaceae species (hereafter 'brassicas') as cover crops to protect the soil from nutrient leaching and erosion (Haramoto and Gallandt, 2004) and to improve the soil structure (Shepherd et al., 2002). However, brassicas are now attracting renewed interest for their potential additional function of being suppressors of soil pathogens. Most brassica species produce sulphur-containing chemical compounds known as glucosinolates (GSLs) (Papavizas and Davey, 1960; Fahey et al., 2001). When plant tissues are damaged, these GSLs are hydrolysed by the enzyme myrosinase, which is stored separately from the GSLs in plant tissues, producing volatile isothiocyanates (ITCs), thiocyanates, water-soluble nitriles and epithionitriles (Brown and Morra, 1997; Kiddle et al., 2001). These hydrolysis products have been shown to suppress plant pathogenic organisms (Muehlchen et al., 1990; Angus et al., 1994; Borek et al., 1996; Lazzeri et al., 2004; Kabouw et al., 2010). These organisms include the soil-borne pathogen *A. euteiches* (Lewis and Papavizas, 1971; Mayton et al., 1996; Smolinska et al., 1997).

The work presented in this thesis sought to evaluate the suppressive effects of brassicas on *A. euteiches* and the development of Aphanomyces pea root rot in subsequent pea plants and to identify possible mechanisms behind the suppression, with the focus on the chemical compounds produced from hydrolysed GSLs in brassica tissues. It also sought to investigate how the brassicas affect some important ecosystem organisms, namely N_2-fixing bacteria and nitrifying organisms.

2 Aims

The overall aim of the thesis was to investigate the mechanisms behind the suppressive effects of brassica cover crops on the development of pea root rot, in order to prevent the threat of the causative pathogen *A. euteiches* in subsequent pea production. An additional aim was to investigate whether the suppressive effects on the pathogen also influenced beneficial N_2-fixing bacteria and ammonium-oxidising organisms.

The following specific objectives applied in **Papers I-IV**:

Paper I. Previous studies on the impact of brassica cover crops on soil organisms and soil quality were reviewed in order to evaluate the factors affecting Aphanomyces pea root rot disease severity, with several controlling strategies, and to analyse in detail the suppressive effects and associated mechanisms of cover crops comprised of different brassica species.

Paper II. The direct effects of incorporating brassica shoot tissues into Aphanomyces-infested soil and the indirect effects of intact growing roots until plant flowering on the development of root rot were investigated by conducting a subsequent bioassay with peas.

Paper III. Results from **Paper II** showing suppression of *A. euteiches* by brassica roots growing in contaminated soil for 11 weeks raised the question of whether beneficial organisms could also be negatively affected. Using soil samples from same greenhouse experiment as in **Paper II**, this study analysed possible changes in the communities of N_2-fixing bacteria and ammonium-oxidising organisms after growth of different cover crops.

Paper IV. In *in vitro* studies, using low temperature dried and milled brassica shoot tissues of two species with different GSL profiles, the effects of volatiles from rehydrated tissues on *A. euteiches* thin-walled mycelium and

thick-walled oospores were investigated. The effects of volatile concentration and duration of exposure and the initial ITC release patterns from the different brassica tissues were also examined.

3 Background

3.1 The pathogen *Aphanomyces euteiches*

Aphanomyces euteiches is not a true fungus due to its cell wall comprising cellulose instead of chitin, but belongs to the oomycetes (*Saprolegniales*) (Petersen and Rosendahl, 2000). Oomycetes have mainly aquatic habitats and have both saprotrophic and parasitic life styles (Baldauf et al., 2000), with both heterothallic (outcrossing) and homothallic (self-crossing) reproductive systems. The male sexual organ (antheridium) fertilises the female organ (oogonium), producing a unicellular spore, the oospore. Oospores can survive in soil without a host plant and remain virulent for 10 to 20 years (Pfender and Hagedorn, 1983). Oospores form a short mycelial germ sporangium and release asexual zoospores. The zoospores are equipped with two flagella and are able to swim in water (*Figure 1*). Zoospores have been shown to have a strong attraction to a diverse range of chemical compounds in order to locate plant roots (Judelson and Blanco, 2005).

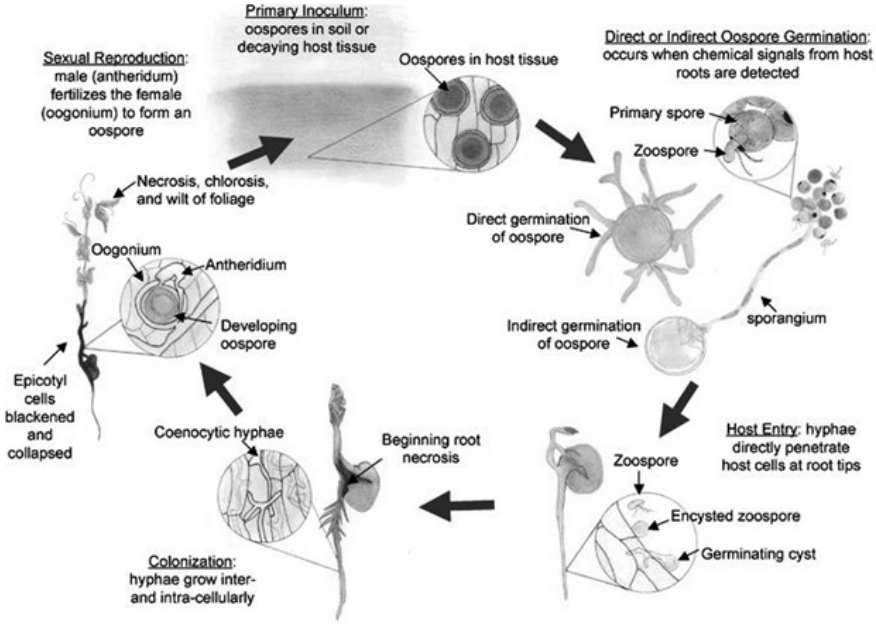

Figure 1. Life cycle of *Aphanomyces euteiches*. Diagram from Hughes and Grau (2007), reproduced courtesy of the American Phytopathological Society.

3.2 Aphanomyces pea root rot

One of the determining problems in pea cultivation is pea root rot caused by *A. euteiches* (Jones and Drechsler, 1925). Pea roots release root exudates such as mucilage and a diverse array of secondary metabolites such as flavonoids (Bowen and Rovira, 1999), which attract *A. euteiches* zoospores and are used as chemical signals to locate favourable infection sites on the pea root (Judelson and Blanco, 2005). The zoospores rapidly shed their flagella and encyst on the host root within minutes. Encysted zoospores can germinate and penetrate into the root cells and form new mycelium within an hour (Papavizas and Ayers, 1974). The mycelium of *A. euteiches* then grows intracellularly through the root tissue and forms large numbers of oogonia, which are fertilised by antheridia and produce new oospores (*Figure 1*).

The main symptom of infection in pea roots is the appearance of water-soaked, brown-coloured soft-rot lesions. Sometimes these lesions allow infection by secondary organisms which destroy the entire root system and the brown-coloured tissue become dark. Symptoms can also appear in the epicotyl region (*Figure 1*). This damage to the root system leads to stunted growth, wilting symptoms and chlorosis of the lower leaves (Hagedorn, 1991).

3.3 Factors affecting pea root rot disease

3.3.1 Soil moisture

Soil moisture is the most important factor for the development of Aphanomyces pea root rot. It provides a favourable environment for oospore germination and promotes zoospore movement. Geach (1936) showed that the development of severity is always favoured by high soil moisture with poor drainage, with 30% soil moisture content seeming to be the minimum for successful infection. In Swedish pea fields, Olofsson (1967) showed that there was no infection when the soil moisture was less than 45% of water-holding capacity. High moisture levels together with humid weather conditions enhance the disease severity (Olofsson, 1967).

3.3.2 Soil temperature

Soil temperature is another important factor for disease development and prevalence. According to Jones and Drechsler (1925), the optimum temperature for infection of a susceptible pea plant by *A. euteiches* is between 15 °C and 30 °C. Smith and Walker (1941) concluded that maximum infection took place when the soil temperature was 24-28 °C, but did not find any infection at 12 °C and below. Lockwood (1960) scored the lowest disease severity at 16 °C and the highest at 28 °C. Yong and King (1963) infested pea root tips with *A. euteiches* oospores and calculated the number of oospores germinating after a suitable incubation time at different temperatures, and found that there was no infection from oospores at 40 °C and that peak infectivity occurred at 20, 25 and 30 °C.

3.3.3 Soil types

Soil type might have some effect on the development and prevalence of Aphanomyces pea root rot. Studies in southern Sweden have shown that pea root rot is a more serious problem in soils with a high clay content (Persson and Olsson, 2000). Densely compacted soil favours pathogen attack on pea roots, since water permeability is low in compacted soil and influences the oospores to release huge numbers of zoospores (Allmaras et al., 2003).

3.4 Control of pea root rot

The thick protective cell walls of *A. euteiches* oospores keep the pathogen viable in soil for a long time without the need for a host plant. However, a

warm, moist environment may stimulate the pathogen to develop, causing disease if a suitable host is present. Lack of *A. euteiches* resistance in pea cultivars and restrictions on fungicide application make pea production very difficult. Therefore, pathogen control strategies have to focus on how to minimise *A. euteiches* infections. There are several approaches available to control Aphanomyces pea root rot, but none of these can persist for a long time and some also have adverse environmental impacts. Applying the fungicide Dexon (sodium p-dimethyl amino benzene diazo sodium sulphonate) to *A. euteiches*-infested soil significantly reduces disease severity (Mitchell and Hagedorn 1971). However, most fungicides inhibit the target pathogen but also beneficial organisms (Johnsen et al., 2001). Combined application of fungicides, lime and synthetic fertilisers may reduce pea root rot, but does not solve the problem entirely (Papavizas and Lewis, 1971; Heyman et al., 2007). Papavizas and Lewis (1971) found that applying $N\text{-}NH_4^+$ fertiliser reduced the disease severity significantly compared with $N\text{-}NO_3^-$ fertiliser. A possible mechanism behind this suppression is that inorganic salts in the fertiliser may increase the osmotic pressure and ionic strength in the soil solution and affect the inoculum of *A. euteiches* (Smith and Walker, 1941; Lewis, 1973). Wade (1955) suggested that the pathogen-suppressing effect of fertilisers is due to more nutrient effects rather than osmotic effects. Another strategy for controlling pea root rot is to rotate the pea crops with non-leguminous crops, where brassica crops are of particular interest (Allmaras et al., 2003; Matthiessen and Kirkegaard, 2006).

3.5 Brassica cover crops

Different brassica species are cultivated as vegetable, oilseed, condiment, forage and medicine crops (Fenwick and Heaney, 1983). Recently, the brassicas have also been introduced as cover crops (Haramoto and Gallandt, 2004), to protect the soil from nutrient leaching and erosion in fallow seasons (Sarrantonio and Gallandt, 2003). In addition, the brassicas improve soil structure and increase infiltration when the crop tissues are incorporated into the soil (Ray et al., 2006). Furthermore, the incorporated tissues release nutrients as they decompose and subsequent crops can utilise these nutrients (Kuo and Jellum, 2002). A more recent interest in brassica cover crops is to control soil-borne pathogens due to the GSL content in their tissues (Brown and Morra, 1997). The hydrolysis products of GSLs have inhibitory effects against a wide range of soil organisms (Fahey et al., 2001), including *A. euteiches* (Papavizas and Lewis, 1971; Stones et al., 2003).

3.6 Glucosinolates in brassica plant tissues

As mentioned, brassica crops produce the sulphur-containing chemicals GSLs (Sang et al., 1984; Fahey et al., 2001). Hydrolysis of these GSLs by myrosinase results in the production of volatile ITCs, thiocyanate (TC) and water-soluble nitriles and epithionitriles (Brown and Morra, 1997; Kiddle et al., 2001). The variety, quantity and distribution of GSLs vary among brassica species and vegetative parts (Fahey et al., 2001; van Dam et al., 2009). Brassica root tissues generally contain higher amounts of GSLs than shoot tissues, but the type of GSLs formed can differ between roots and shoots (Rosa, 1997; van Dam et al., 2009). Glucosinolates in brassica tissues can be classified into three different types, aliphatic, aromatic and indolyl GSLs, distinguished on the basis of their organic chemical structure (Wittstock and Halkier, 2002).

3.7 Bio-fumigation

The bio-fumigation concept in agricultural systems was introduced by Australian scientists in the 1990s and includes practices to suppress soil-borne pests and pathogens by the use of brassica crops (Angus et al., 1994). The volatile ITCs produced from hydrolysed GSLs in brassica plant tissues have been shown to inhibit soil organisms, by acting similarly to the synthetic soil fumigant methyl bromide (Matthiessen et al., 1996). In bio-fumigation, the brassica crops are grown until flowering, whereupon they are cut, chopped and incorporated into the upper soil layer. To optimise the hydrolysis process and the production of volatiles, irrigation is generally used. The inhibitory effect of ITCs depends on their type (Smolinska et al., 2003) and concentration (Angus et al., 1994). Aliphatic ITCs usually have stronger inhibitory effects than aromatic ITCs (Smolinska et al., 2003; Matthiessen and Shackleton, 2005). This difference is due to their differing mode of action (Kawakishi and Kaneko, 1985; Kirkegaard et al., 1998), leading to different types of ITCs exerting different degrees of toxicity at a particular concentration (Smith and Kirkegaard, 2002). According to Kawakishi and Kaneko (1985), aliphatic ITCs bind with protein molecules inside the cells and block them, collapsing the cells, while aromatic ITCs interact with the electrophilic properties of the cell membrane and damage the cells (Abreu et al., 2013). The toxicity of the ITCs also depends on the size of the organic group attached, with toxicity decreasing with size (Falk et al., 2004; Yuesheng, 2012). The effect of a certain ITC can also differ depending on the species of organism exposed (Angus et al., 1994).

3.8 Bio-fumigation in disease control

It has been shown that the hydrolysis products of GSLs can inhibit *A. euteiches* and reduce Aphanomyces pea root rot. Lewis and Papavizas (1970) demonstrated in *in vitro* studies that volatile products from decomposing cabbage tissues inhibit hyphal growth of *A. euteiches*. Those authors also observed the treated pathogen was unable to grow when placed in fresh air. Dandurand et al. (2000) showed that the volatile hydrolysis products of rapeseed meal (*Brassica napus*, cv. Dwarf Essex, high GSL content) strongly inhibited the soil-borne pathogens *Sclerotinia sclerotiorum* and *A. euteiches*. They also found that the volatile products completely prevented *S. sclerotiorum* sclerotia germination and prevented hyphal growth of *A. euteiches* by 77% (Dandurand et al., 2000). However, both pathogens were unaffected by low GSL content in *B. napus* (cv. Stonewall) meal. A greenhouse study showed that incorporating cabbage tissue can significantly reduce pea root rot (Lewis and Papavizas, 1971). Mazzola et al. (2001) found in a greenhouse experiment that soil amended with seed meal of *B. napus* (cv. Dwarf Essex) suppressed apple replant disease (causal agents *Rhizoctonia solani* and the nematode *Pratylenchus penetrans*). In a field study, a *Sinapis alba* (white mustard) cover crop significantly reduced Aphanomyces pea root rot in subsequent peas after incorporation of the white mustard tissues into *A. euteiches*-contaminated soil. On the negative side, brassica tissues and crops causing suppression of soil-borne pathogens may also suppress beneficial and non-target organisms in the soil, such as N_2-fixing and nitrifying organisms. Muehlchen et al. (1990) studied the formation of pea root nodules when peas were grown in soil with brassica tissues incorporated and suggested that GSL hydrolysis products could inhibit nodule-forming rhizobium bacteria. Bending and Lincoln (2000) proved that exposure to synthetic volatile ITCs inhibits soil nitrifying bacteria. The mechanisms behind the suppression can be related to GSLs hydrolysis products and/or to N nutrients released after incorporation of the brassica tissues into the soil (Papaviza and Lewis, 1971; Matthiessen and Kirkegaard, 2006).

4 Methods

4.1 Pilot study

An initial pilot study was conducted to select cover crops affecting Aphanomyces pea root rot in subsequent pea plants for further experiments. Commercial garden soil was inoculated with *A. euteiches* oospores (800 oospores/mL soil). The cover crops *Brassica juncea*, *Sinapis alba*, *Raphanus sativus*, *Secale cereale* and *Lolium multiflorum* were then grown separately in inoculated soil or in a control (bare soil) for 9 weeks, until the brassica crops had reached the flowering stage. Root and shoot tissues of the cover crops were harvested, macerated and incorporated into *A. euteiches*-inoculated soil. Pea seeds were sown in the tissue-amended soil and incubated in the greenhouse for 5 weeks (bioassay).

The results from the pilot study was statistically analysed in accordance with its completely randomised design, with treatments as fixed factor and replicates as random factor. The effect of cover crops as factor and replicate as random factor fitted a linear model (lm). The least square means were compared using the Honestly Significant Difference (HSD) Tukey's test, with $P<0.05$ significance limit, in R version 2.15.1 (The R Foundation, 2012).

4.2 Bioassay

A pea bioassay was used to evaluate the effect of oospores in differently treated field soils in **Papers II-IV**. The assay was carried out in the greenhouse using the root rot-susceptible pea cultivar Clare and included 4 weeks (**Papers II and III**) or 3 weeks (**Paper IV**) of incubation, compared with 5 weeks in the initial pilot study. After the incubation period, the pea plant roots were carefully washed and the disease symptoms were recorded for individual plants. A disease severity index (DSI) value based on the mean disease

symptoms was assigned to each pot of plants. The scale for DSI ranged from 0 to 100 (%), but each individual plant could only be assigned one of five scores for disease severity: 0% = healthy plant; 25% = root slightly discoloured; 50% = root extensively discoloured but not shrunken; 75% = root extensively discoloured and shrunken; and 100% = root partly or completely rotted or plant dead (Parke et al., 1991). The DSI scale is illustrated in *Figure 2*.

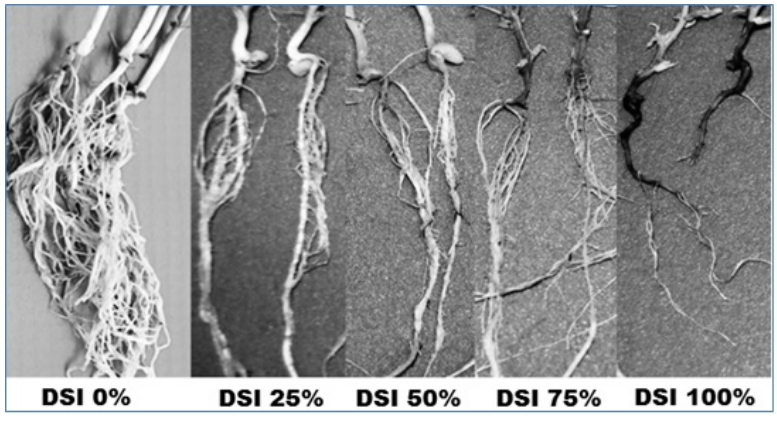

Figure 2. Scale used for the Aphanomyces pea root rot disease severity index (DSI).

4.3 Effect of volatile compounds on *A. euteiches* in *in vitro* and *in vivo* experiments

Papers II and IV examined the effects of volatile ITC compounds on *A. euteiches* mycelium and oospores. A test system consisting of a 220-mL plastic cup with the brassica tissues in the bottom and the *A. euteiches* Petri dish culture placed on top as a lid was developed (*Figure 3*A). Talcum powder containing oospores was placed in a plastic sieve, a plastic cup was placed as a lid on top of the sieve and the combined unit was placed on top of a cup containing brassica tissues (*Figure 3*B). The cup had the same diameter as the Petri dish, and cup and dish were sealed with several layers of clingfilm. It was thus possible to measure the *A. euteiches* growth on the lid without opening the seal.

Figure 3. Volatile compounds from brassica shoot tissues exposed to (A) a culture and (B) oospores of *Aphanomyces euteiches* (placed on top of the plastic cup).

In **Paper IV**, oospores that had been exposed to volatiles from different brassicas were mixed with sterilised soil and tested for 3 weeks in a pea root rot bioassay. Growth of 3-week-old pea plants in the oospore-amended soil in the different treatments is shown in *Figure 4*.

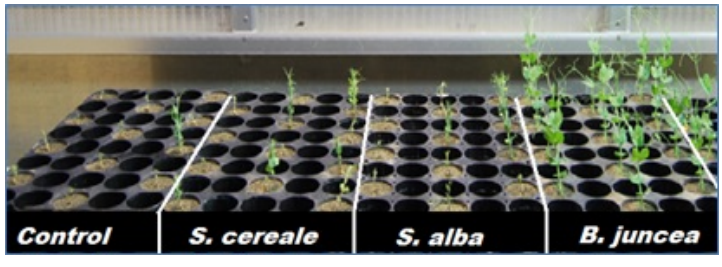

Figure 4. Effects of volatile compounds on oospores pre-treated with volatiles from different brassica in terms of their ability to cause Aphanomyces root rot in peas (3-week bioassay).

4.4 Analysis of volatile compounds

In **Paper IV**, volatile compounds produced from hydrated brassica tissue were detected and quantified by gas chromatography-mass spectrometry (GC-MS). The powdered brassica tissue was placed in a glass plate in a bell-shaped glass vessel (380 mL) with inlet and outlet openings sealed with bulldog clips. Adding water to the tissue immediately produced volatile compounds, which were collected by drawing air from the outlet of the jar through a glass liner containing Tenax TA during 130 min of tissue GSL hydrolysis (*Figure 5*). The types and quantities of GSLs in *B. juncea* and *S. alba* shoot powder were analysed by high-performance liquid chromatography (HPLC).

Figure 5. Equipment used for collecting volatile compounds from hydrated brassica tissue powder. The compounds were removed by drawing air from the outlet of the jar and conducted to analysis equipment via a tube (bottom).

In **Paper II**, volatile compounds were collected from the soil rhizosphere environment of *B. juncea* and *S. alba* cover crops after 11 weeks of growth in *A. euteiches*-infested soil (*Figure 6*). A hole was made vertically in the growing boxes 5 cm below the soil surface and a 5 cm long glass tube was inserted. Volatile compounds were collected by drawing air from the hole for 24 h and were analysed by using GC-MS.

Figure 6. Equipment used for collecting volatile compounds from the soil rhizosphere of brassica cover crops.

4.5 Real-time PCR for the detection and quantification of N_2-fixing and nitrifying organism communities

Paper III investigated the possible effects of brassica plants on soil N_2-fixing bacteria and ammonia-oxidising organisms. For this purpose, the quantitative real-time PCR method was used. The effect on the soil microbial communities was estimated by quantifying gene copies of key enzymes involved in N_2 fixation and ammonia oxidation. DNA was extracted from differently treated soil samples. The numbers of *nifH* gene copies of N_2-fixing bacteria and of *amoA* gene copies of nitrifying organisms such as ammonium-oxidising archaea and bacteria were quantified, giving an estimation of the community size of the respective organism.

5 Results and discussion

5.1 Pilot study

The initial pilot study showed that incorporation of brassica plant tissues grown for nine weeks in *A. euteiches*-infested soil significantly ($P<0.001$) reduced root rot severity in subsequent pea plants compared with the non-brassica cover crops (*Figure 7*).

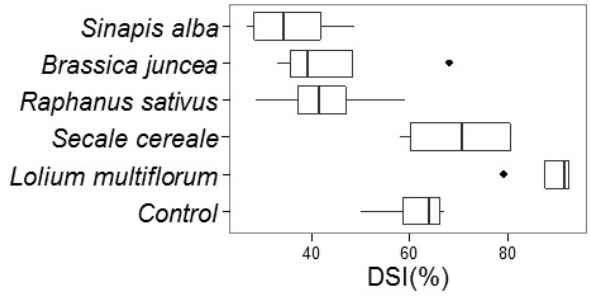

Figure 7. Effects of different cover crops grown for nine weeks in an *A. euteiches*-infested soil on the development of root rot in the subsequent pea plant bioassay. DSI = disease severity index

The two brassica crops (*Brassica juncea* and *Sinapis alba*) showed the best suppressive effects against the development of pea root rot (*Figure 8*). Their suppressive effects, in conjunction with the fact that these two species have different GSL profiles, were the basis for choosing them for further studies. *Secale cereale* was chosen as a non-GSL producing cover crop to be included in further experiments.

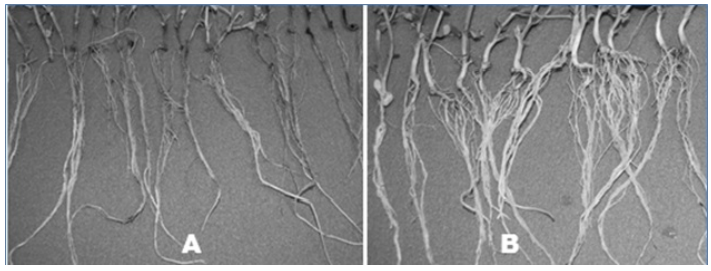

Figure 8. Pea root rot disease severity (A) without a cover crop and (B) with a brassica cover crop in peas grown for five weeks in a bioassay.

5.2 Effects of volatile compounds from brassica shoot tissue on *A. euteiches in vitro* and *in vivo*.

The volatile compounds developed from fresh macerated (**Paper II**) and dry, rehydrated *B. juncea* shoot tissue significantly suppressed hyphal growth and reduced the ability of oospores to infect plants compared with volatiles from *S. alba* shoot tissues (**Paper IV**). However, fresh macerated shoot tissues of *B. juncea* incorporated into *A. euteiches*-infested soil were unable to reduce pea root rot when peas were sown directly after incorporation (**Paper II**). The volatile compounds were probably quickly lost in this case and left the pathogen unaffected. In fact, since we used a structured clayey soil, it is possible that the majority of the ITCs produced passed through the major pores, leaving oospores of *A. euteiches* unaffected in smaller pores and within the soil aggregates. The toxic effects could have been enhanced by sealing the soil surface immediately after incorporation, thereby preventing the ITCs from being lost, as we saw a clear suppressive effect on the pathogen in the *in vitro* closed environment experiment.

Analysis of glucosinolates and isothiocyanates of *B. juncea* and *S. alba* shoot tissues showed that the aliphatic GSL sinigrin dominated in the *B. juncea* tissue and the aromatic GSL sinalbin dominated in *S. alba* tissue (**Paper IV**). The ITC analysis showed accordingly that the dominant hydrolysis compounds detected were aliphatic allyl ITC and aromatic benzyl ITC. Previous studies have shown that aliphatic allyl ITC is more toxic to soil-borne pathogens than aromatic benzyl ITC (Smolinska et al., 2003; Matthiessen and Shackleton, 2005). The investigations in **Paper IV** showed that the effectiveness of a specific ITC and a sufficient concentration are not enough to inhibit the pathogen and that the duration of exposure is another essential factor for a suppressive effect.

5.3 Growth of cover crops in *A. euteiches*-infested soil – effects on pea root rot, N_2-fixing and nitrifying organism communities

Growth of brassica cover crops for 11 weeks in *A. euteiches*-infested soil significantly reduced Aphanomyces pea root rot in subsequent bioassayed pea plants, e.g. as observed in the *S. alba* treatment ($P<0.002$) (**Paper II**, *Figure 9*). Analysis of the volatile compounds collected from the brassica rhizosphere in the soil environment after 11 weeks of brassica plant growth showed that GSL root exudates of *S. alba* produced a higher variety and quantity of aliphatic ITCs than were detected in the *B. juncea* root-soil environment (**Paper II**). The suppressive effect on root rot observed for the *S. alba* treatment may have originated from the toxic aliphatic ITCs detected being present in higher concentrations than measured for the *B. juncea* root-soil environment.

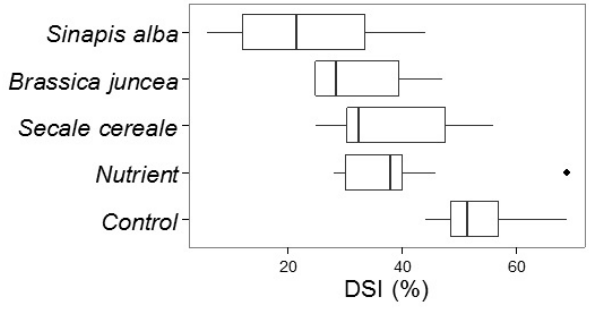

Figure 9. Effects of different cover crops on the development of Aphanomyces pea root rot in subsequent pea plants. Disease severity index (DSI) values were back-transformed from the natural logarithm values used in the statistical analysis.

Paper III showed that growing brassica roots did not reduce the abundance of soil N_2-fixing and nitrifying organism communities (*Figure 10*). Earlier studies had suggested that the hydrolysis products of GSLs show inhibitory effects on the abundance of N_2-fixing and nitrifying organism communities (Bending and Lincoln, 2000). The ITCs detected in the brassica root-soil environment in **Paper II** did not affect the N_2-fixing and ammonia-oxidising organism communities negatively. It has also been shown previously that different soil organisms show different levels of sensitivity to specific ITCs and that organisms require a certain concentration of specific ITCs for potential suppression (Angus et al., 1994; Sang et al., 1984). The test system used in **Paper III** did not seem to produce ITCs affecting these organisms, or else they

were produced in a lower concentration than were inhibitory for these organism communities.

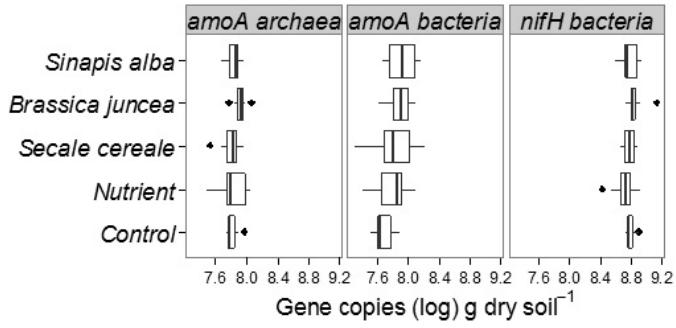

Figure 10. Abundance of *amoA* gene of ammonium-oxidising archaea and bacteria and *nifH* gene of N_2-fixing community (copies/g dry soil) in soil sampled after 11 weeks of cover crop growth and four weeks of pea bioassay.

6 Conclusions

Based on the results presented in **Papers II-IV**, it was possible to draw the following conclusions:

1. Volatile compounds developed from *B. juncea* shoot tissue strongly inhibit the pathogen *Aphanomyces euteiches*, at least in a closed system studied *in vitro*.

2. The volatile compounds released from *B. juncea* shoot tissue material after incorporation into infested soil did not suppress the development of Aphanomyces pea root in a subsequent bioassay where peas were sown directly after the incorporation.

3. In a potential bio-fumigation process, important factors are the choice of brassica crop, reaching a sufficient concentration of volatile ITCs and exposure for a sufficient period of time. This indicates a need for soil covering after tissue incorporation.

4. Growth of brassica species, especially *Sinapis alba* (white mustard) significantly reduces Aphanomyces root rot disease severity in subsequent pea plants. Aliphatic isothiocyanates detected in the soil-rhizosphere environment in our studies may be an inhibiting factor.

5. Brassica cover crops with two different GSL profiles grown for 11 weeks in soil did not reduce the abundance of soil N_2-fixing and nitrifying organism communities. The amount of volatile compounds produced was not enough or they were not of the right type to prevent the growth of such communities.

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