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Oskar Björling

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Oskar Björling

Supervisor: Dr Arne Schwelm, Swedish University of Agricultural Sciences, Department of Plant Biology and Forest Genetics, Uppsala
Assistant Supervisor: Prof. Christina Dixelius, Swedish University of Agricultural Sciences, Department of Plant Biology and Forest Genetics, Uppsala
Examiner: Dr Anders Kvarnheden, Swedish University of Agricultural Sciences, Department of Plant Biology and Forest Genetics, Uppsala

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Abstract

In this thesis, three separate experiments have been performed on different aspects of the interaction between the causal agent of clubroot, *Plasmodiophora brassicae*, and its hosts.

In the first experiment the pathotype of the *P. brassicae* single spore isolate, which is currently used to construct a reference genome, has been classified using the ECD bioassay. The disease severity of infected plants was scored according to two different scales and the pathotype was determined according to three previously published guidelines. The results were compared to previous published studies describing the e3 isolate.

The life cycle of *P. brassicae* is not well understood. A small-scale cultivation system was tested to study the early interactions between *P. brassicae* and host plants on a molecular basis using DNA and RNA analyses from plant roots and rhizosphere. Infection rates were determined by quantification of *P. brassicae* DNA in root samples. DNA and RNA extractions from plant roots were successful but the system did not provide consistent infection. Disease development rates and gene expression analyses did not generate reliable results.

The third experiment was a pilot study to investigate the influence of root exudates from inoculated and non-inoculated plants on *P. brassicae* resting spore germination behaviour. Root exudates of several host and non-host plants are known to stimulate resting spore germination under experimental conditions but the effect of non-host plants is often diminished under field conditions. It was tested if the infection of the plant roots by *P. brassicae* could influence the germination of resting spores in the environment. The results indicated that infected roots may increase resting spore germination. The effect seemed to vanish as the *P. brassicae* infection reached later stages in its lifecycle.

Sammanfattning

Klumprotsjuka är en allvarlig sjukdom som angriper oljeväxter. Den orsakas av den obligata och biotrofa patogenen *Plasmodiophora brassicae*. I detta examensarbete ingår tre separata experiment som alla syftar till att undersöka olika aspekter av interaktionen mellan *P. brassicae* och dess värdväxter.

I det första experimentet undersöktes aggressiviteten av ett *P. brassicae* enkelsporisolat, e3, på 15 olika värdväxter som odlades i växthus. Aggressiviteten av isolatet bedömdes genom att gradera symptomen på värdväxternas rötter för att bestämma isolatets patotyp. Informationen var av särskilt stor betydelse eftersom isolatet använts i flera tidigare och pågående studier, bl.a. för utvecklingen av ett referensgenom. Alla 15 värdväxterna utvecklade mer eller mindre kraftiga symptom vilket visar att e3 isolatet är ett aggressivt isolat med vid värdkrets.

Det andra experimentet utfördes för att studera interaktionen mellan patogenen och värdväxten på gennivå med hjälp av kvantitativ PCR–teknik. Att mäta vilka gener som uttrycks under infektionsförloppet är en vanlig teknik för att studera processer och regulatoriska nätverk under sjukdomsförloppet. Kunskap inom området är en nyckel i forskning och utvecklingen av nya sätt att kontrollera sjukdomen. För att studera genuttrycket under infektionsprocessen utvecklades ett enkelt odlingssystem där växter kunde odlas och inokuleras med *P. brassicae* under standardiserade förhållanden. DNA och RNA extraherades ur växternas rötter och ur jorden. DNA och RNA från växternas rötter höll tillräckligt god kvalitet för att analyseras. Patogenens tillväxt mättes i förhållande till växtens genom via kvantifiering av DNA. Analysen indikerar ojämn sjukdomsutveckling mellan individuella replikat vilket tyder på att odlingssystemet inte fungerade optimalt. Resultaten av genuttrycksanalysen bedömdes inte som tillförlitliga.

P. brassicae bildar långlivade vilsporer vilket gör att smittan kan finnas kvar i marken under lång tid efter ett sjukdomsutbrott även i avsaknad av värdväxter. Flera studier har visat att groningen av vilsporer sker spontant, men att ett flertal biotiska och abiotiska faktorer kan påverka sporernas groning. Att utnyttja mekanismen som styr groningen för att stimulera denna utan att patogenen ges möjlighet att slutföra sin livscykel efter groningen är en av två huvudsakliga strategier som har föreslagits för biologisk kontroll av *P. brassicae*. Ett flertal försök med att använda olika växter för att inducera sporgroning har tidigare utförts med blandade resultat. I den tredje delen av det här examensarbetet genomfördes en pilotstudie för att undersöka om rotexudat från inokulerade rötter har större groningsstimulerande effekt på vilsporer jämfört med rotexudat från friska plantor. Resultaten visar att rotexudat från inokulerade rötter stimulerar vilsporgroningen mer än friska rötter och att den stimulerande effekten varierar beroende på vilket stadium av sjukdomen som förekommer i växtrötterna när rotexudaten utsöndras. Ytterligare forskning inom området behövs för att nå en djupare förståelse för vilka faktorer som styr groningen hos vilsporerna och hur dessa kan utnyttjas för att utveckla nya produkter för sjukdomsbekämpning.

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1. Introduction

Background

Clubroot is a severe disease of oilseed rape, cabbages, oilseed turnip (Hwang *et al.* 2012a) and all the other ~3700 potential host plant species belonging to the *Brassicaceae* family (Dixon 2009a). It may cause total yield loss in infested fields (Hwang *et al.* 2012a). A study by Crête (1981), on the worldwide importance of clubroot, indicates that crop losses caused by clubroot corresponds to the general estimation of in-field crop losses caused by individual crop pathogens of 10-15% (Dixon 2009a). The pathogen causing clubroot, *Plasmodiophora brassicae*, is commonly occurring in Swedish fields (Wallenhammar 1996). Increased cultivation of Brassica crops for oilseed production and other uses (Fig. 1) has led to increased clubroot incidence and yield losses due to clubroot in Sweden and many other parts of the world (Donald and Porter 2009, Wallenhammar *et al.* 2012).

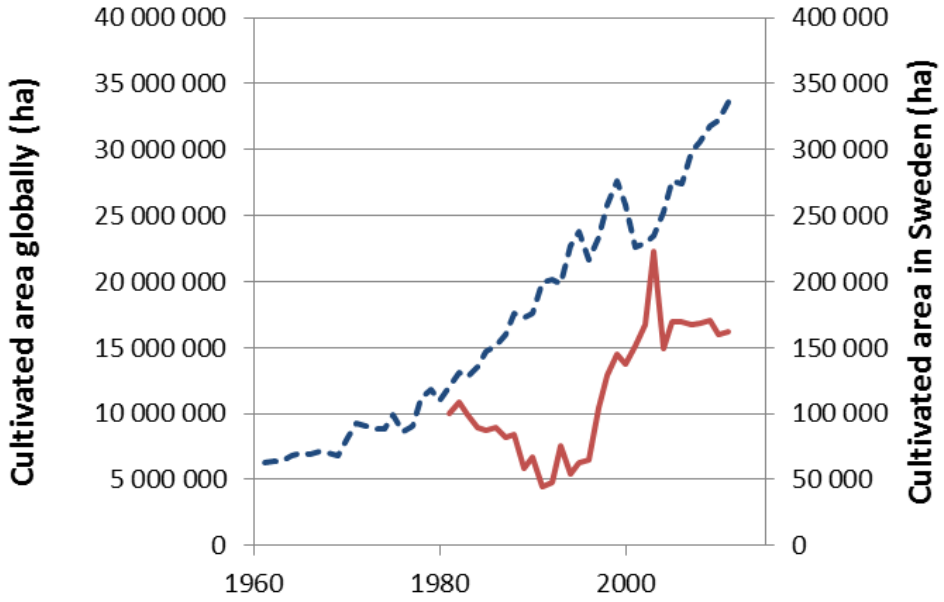

Fig. 1. Increase of global (- - -) and Swedish (——) oilseed rape cultivation area (FAOSTAT 2013, Swedish Board of Agriculture 2013).

P. brassicae is an obligate biotrophic protist (Kageyama and Asano 2009) belonging to the eukaryotic supergroup Rhizaria (Burki *et al.* 2010). The lifecycle of *P. brassicae* (Fig. 2) involves three distinct stages of which two take place inside the host (Tommerup and Ingram 1971, Kageyama and Asano 2009). In the first stage, resting spores in the soil release primary zoospores. In presence of susceptible plants the zoospores penetrate the root hairs and develop into primary plasmodia. This infection stage does not cause any obvious macroscopic symptoms of infection, but results in a slightly increased plant growth rate (Devos *et al.* 2005). The primary plasmodium develops into zoosporangium, and secondary zoospores are released approximately 7-8 days after infection (Tommerup and Ingram 1971). The secondary zoospores and the primary zoospores cannot be visually differentiated. Observations of binucleate zoospores have led to the assumption that fusion of zoospores can occur.

Secondary zoospores infect the cortical tissues of the plant roots and develop into secondary plasmodia (Kageyama and Asano 2009).

The development of the secondary plasmodia in the cortical tissue constitutes the second stage of the pathogen's lifecycle. The secondary plasmodia undergo nuclear division and develop into multinuclear plasmodia. It is hypothesized that genetic recombination occurs in the secondary plasmodia through fusion of two haploid nuclei in multinuclear plasmodia followed by subsequent meiotic cleavage (Kageyama and Asano 2009). This hypothesis is although not universally accepted (Fähling *et al.* 2004). This secondary infection leads to cellular hypertrophy, altered biochemistry, physiology and metabolic patterns within the host (Devos, *et al.* 2005, Feng *et al.* 2012) resulting in the typical macroscopic symptoms associated with clubroot i.e. clubbed roots, wilting, premature death and reduced yield (Howard *et al.* 2010). The secondary infection stages have a narrower host range compared to the primary infection stages which occur with non-susceptible plants (Ingram and Tommerup 1972, Friberg *et al.* 2005).

At the end of the secondary infection stage, resting spores are produced inside the secondary plasmodia (Kageyama and Asano 2009). These resting spores are then subsequently released into the soil as the plant tissue decays. The resting spores and their survival in the soil constitute the third part of the lifecycle. The resting spores are very persistent with an estimated half-life of 3.6 years in soil. It can take more than 17 years until the population has decreased below detectable levels in infested soils using a bioassay method (Wallenhammar 1996). Resting spore germination and the release of zoospores occur spontaneously but can be influenced by various environmental factors, e.g., by the presence of certain plant species as bait crops (Friberg *et al.* 2005).

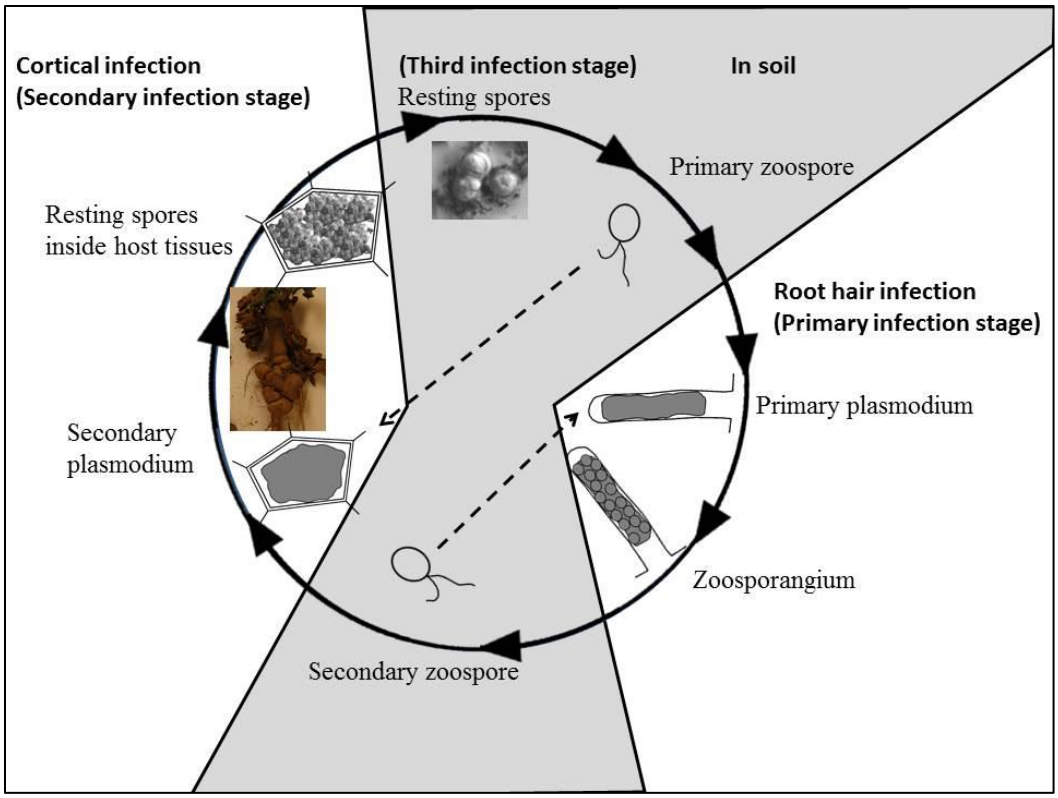

Fig. 2. Illustration of the *P. brassicae* lifecycle, modified after Kageyama and Asano (2009).

Despite a severe nature of the clubroot disease and having it as a historically well-researched disease, the biology of *P. brassicae* and the disease control remain partly unknown. The obligate biotrophic nature of *P. brassicae* makes it very inconvenient to study (Feng *et al.* 2012). The possibilities to control the disease are very limited as there is currently no effective, safe or affordable method or commercial product capable of achieving satisfying control of clubroot on a field scale once disease has been established in a field (Deora *et al.* 2012, Donald and Porter 2009). Some proactive measures can be employed by farmers in order to disfavour *P. brassicae* and avert clubroot disease outbreak e.g. liming, drainage, weed management of Brassica weeds (including volunteers), plant nutrition and crop rotation (Dixon 2009b, Donald and Porter 2009). Using resistant cultivars is by far the most successful strategy to control the disease and creating durable resistance against clubroot has been frequently attempted. There are however many examples of rapid adaptations of *P. brassicae* populations to overcome the resistance genes used (Hatakeyama *et al.* 2004, Oxley 2007, Jubault *et al.* 2008). This gene erosion can be explained by a strong selection pressure imposed by *P. brassicae* populations (Buczacki *et al.* 1975, Strelkov *et al.* 2007, Hwang *et al.* 2012b).

The apparent lack of effective measures to avoid yield losses caused by *P. brassicae* implies a great need for research to develop new products and strategies to control the disease. Insufficient knowledge in the areas of plant-microbe interactions, biology, biochemistry and genetics has been frequently cited as an impediment to the development of new control measures. Advances in these areas will hopefully provide new insights on how to reduce yield losses caused by *P. brassicae* (Werner *et al*. 2008, Diederichsen *et al*. 2009, Feng *et al*. 2010).

Problem definition

P. brassicae is causing substantial problems in the production of cruciferous crops all over the world. Development of counter measures against the disease is slow due to limited knowledge about the pathogen, partly due to its obligate lifestyle which makes studies on this organism difficult. Therefore, improved methods to study, and/or improved knowledge regarding plant-pathogen interactions, biology, genetics and biochemistry are of great significance.

Limitations

The research carried out during this master thesis was adapted to fit into the limited time-frame of a master thesis and the literature study is solely conducted in order to give background and support the materials and methods used and the results obtained from the thesis research.

2. Classification of the *P. brassicae* e3 isolate

2.1 Introduction

Different isolates of *P. brassicae* appear to have different host preferences, or physiological specialization (Buczacki *et al.* 1975, Strelkov *et al.* 2007, Hwang *et al.* 2012b). Populations with specific host preferences are referred to as races or pathotypes. Up to date the *P. brassicae* pathotypes are determined by different bioassay methods (Hatakeyama *et al*. 2004). One of the most common bioassay methods is the European clubroot differential (ECD) bioassay test (Buczacki *et al.* 1975). The ECD test has been used in many previous studies and has been regarded as the standard method for pathotype determination of *P. brassicae* isolates over the last decades (Vorrips 1996, Hatakeyama *et al*. 2004, Strelkov *et al*. 2007). This particular bioassay consists of 15 different genotypes representing three subsets of three different species: *Brassica rapa, B. napus* and *B. oleracea*. These genotypes represent different combinations of clubroot resistance (*CR*) genes. The exact information about the number and identity of these resistance genes are not known but it is assumed that each genotype contains a complex of pathotype specific *CR* genes (Diederichsen *et al*. 2009). The ECD test is sometimes referred to as insensitive since combinations of *CR* genes are known to result in fewer compatible interactions (Strelkov *et al.* 2006, Matsumoto *et al*. 2012). Other plant genotypes can be included in the bioassay to detect differences in virulence between isolates (Hatakeyama *et al*. 2004). Pathotype determination of *P. brassicae* field populations is essential for management of clubroot resistant (*CR*) varieties. Field populations often consist of a complex of pathotypes (Buczacki *et al.* 1975, Fahling *et al.* 2003, Strelkov *et al.* 2007, Hwang *et al.* 2012b). Hence, the use of single race-specific resistance genes against *P. brassicae* imposes strong selection pressure for virulent pathotypes. Using isolates derived from a single resting spore has been a way for researchers to reduce the genetic diversity in *P. brassicae* populations, providing a more uniform material to work with (Tingal and Webster 1981, Voorrips 1996). The e3 isolate, used in this thesis, is a single spore isolate (Fähling *et al*. 2003, 2004). A genome sequencing project at the Swedish University of Agricultural Sciences (SLU) has generated a genome sequence draft of the e3 isolate, which will be the first sequenced plasmodiophorid organism (Schwelm *et al.* unpublished). However, the pathotype of the e3 isolate was unknown. The determination of the race of the e3 isolate is essential for initiating further research aiming to uncoil the largely unknown relationships between genotype and phenotype of *P. brassicae* using the e3 isolate as a reference genome.

Aim

The aim of this experiment was to determine the pathotype of the *P. brassicae* e3 isolate using the ECD bioassay (Buczacki *et al*. 1975).

Hypothesis

The e3 isolate will produce disease of varying severity on the host set included in the ECD test.

2.2 Materials and methods

The seeds of the 15 ECD hosts (Table 1) were provided by the University of Warwick, UK (Warwick Genetic resource centre). *Brassica napus* var. *oleifera* cv. Mendel and cv. Express were obtained from Lantmännen Lantbruk/SW Seed.

Table 1. The European Clubroot Differential (ECD) series: host species with binary values, reproduced after Buczacki *et al*. (1975).

Differential Number	Differential Host		Binary Value
	Cultivar Line	Other Name	
20 chromosome group (*Brassica rapa*)			
1	var. *rapifera* line aaBBCC	Fodder turnip	1
2	var. *rapifera* line AAbbCC	Fodder turnip	2
3	var. *rapifera* line AABBcc	Fodder turnip	4
4	var. *rapifera* line AABBCC	Fodder turnip	8
5	var. *chinensis* cv. Granaat	Chinese cabbage Pe-Tsai	16
38 chromosome group (*Brassica napus*)			
6	var. *napus* line Dc101	Fodder rape Nevin	1
7	var. *napus* line Dc119	Giant rape commercial	2
8	var. *napus* line Dc128	Giant rape selection	4
9	var. *napus* line Dc129	New Zealand resistant rape	8
10	var. *napus* line Dc130	Swede Wilhelmsburger	16
18 chromosome group (*Brassica oleracea*)			
11	var. *capitata* cv. Badger Shipper	Cabbage	1
12	var. *capitata* cv. Bindsachsener	Cabbage	2
13	var. *capitata* cv. Jersey Queen	Cabbage	4
14	var. *capitata* cv. Septa	Cabbage	8
15	var. *fimbriata* cv. Verheul	Fimbriate kale	16

Clubroots of *Brassica rapa* var *chinensis* cv. Granaat inoculated with *P. brassicae* e3 isolate were obtained from Ludwig-Müller. Resting spores were extracted and purified using an in house developed adaption of the protocol described by Asano (1999) and Schwelm, (unpublished).

Host infection and ECD test

A total of 33 plants of each ECD host were grown in 11 pots containing moist 50:50 (volume) Hasselfors garden S soil and Rådasand 0.50 mm special sand mixture. The plants were watered daily with tap water to keep the soil moisture near field capacity. The greenhouse conditions were set at 16 h light and 8 h dark and the temperature ranged between 16 – 22° C.

Surface sterilised resting spores were adjusted to a concentration of 10^7 spores/ml using a light microscope and haemocytometer. Six days after planting the seeds; three 2.5 cm deep holes were punched near the base of the stem of each seedling. One ml of spore solution containing 10^7 spores, was equally divided into the three previously punched holes pointing toward the root system of eachseedling. Three plants were mock inoculated with one ml sterile water as a control. After inoculation the holes were covered with soil and the procedure was repeated the following day. Plants were harvested at 49 days post-inoculation (DPI) to determine the infection level. The soil was carefully removed and the roots were rinsed in tap

water and the symptoms were visually assessed to determine the disease severity on a six-graded scale ranging from 0 - 5, where 0 = no galls; 1 = enlarged lateral roots; 2 = galls on the tap root; 3 = enlarged napiform tap root but healthy lateral roots; 4 = enlarged napiform tap root, few lateral roots infected (small clubs); 5 = enlarged napiform tap root, most of the lateral roots infected, as described by Crête *et al*. (1963). The average disease severity index (DSI) was calculated according to Crête *et al.* (1963) as described by Wallenhammar *et al*. (2000).

$$DSI = \frac{\sum(n \cdot 0 + n \cdot 1 + n \cdot 2 + n \cdot 3 + n \cdot 4 + n \cdot 5)}{total\ number\ of\ plants \cdot 5}$$

Where n is the number of plants in each class and 0, 1, 2, 3, 4 and 5 are the symptom severity classes.

Disease severity was also assessed using a four graded scale ranging from 0 - 3, where 0 = no galling; 1 = a few small galls; 2 = moderate galling, and 3 = severe galling, developed by Kuginuki *et al.* (1999). DSI was calculated using a modified formula from Horiuchi and Hori (1980) as described by Strelkov *et al.* (2006).

$$DSI = \frac{\sum(n \cdot 0 + n \cdot 1 + n \cdot 2 + n \cdot 3)}{total\ number\ of\ plants \cdot 3}$$

Where n is the number of plants in each class and 0, 1, 2 and 3 are the symptom severity classes.

Triplet codes were generated using the binary values, a system used internationally for the ECD hosts, where the binary numbers of all susceptible hosts in each chromosome group are added.

2.3 Results

The upper parts of the plants did not show clear symptoms of stunted growth and the difference between inoculated plants and healthy controls was not obvious at 49 DPI.

The DSI of the 15 ECD hosts used varied between 36 and 95 when symptoms were assessed (Crête *et al.* 1963) as described by Wallenhammar (2000). Only ECD host number 11 (*Brassica oleracea* var. capitata cv. Badger Shipper) had a DSI below 50 (Fig. 3 and 4).

The DSI of the 15 ECD hosts used varied between 49 and 99 when symptoms were assessed according to the four graded scale (Kuginuki *et al.* 1999) and DSI was calculated using the formula modified from Horiuchi and Hori (1980) and Strelkov *et al.* (2006).

Fig. 3 *P. brassicae* e3 isolate inoculated roots of *Brassica oleracea* cv. Badger Shipper (upper picture), mock-inoculated roots (middle picture) of *Brassica oleracea* cv. Badger Shipper and inoculated roots of *Brassica oleracea* var. *capitata* cv. Septa (lower picture) at 49 DPI.

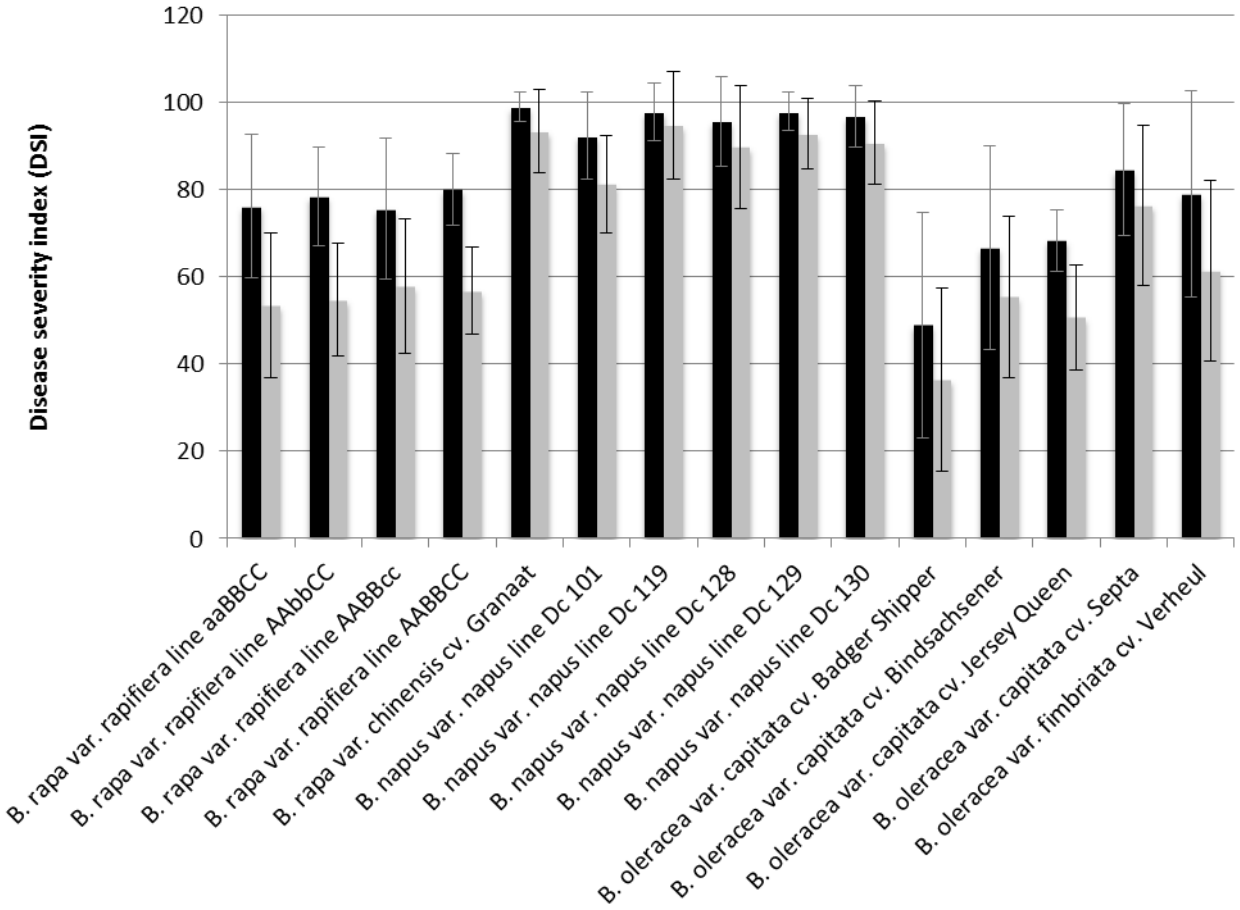

Fig. 4. Average disease severity index (DSI) and standard deviation of the 15 ECD hosts (Table 1) inoculated with $2 \cdot 10^7$ spores of the e3 isolate per plant assessed at 49 DPI according to the instructions by Kuginuki *et al.* (1999), Strelkov *et al.* (2006) (black) and Crête *et al.* (1963) (grey).

2.4 Discussion

The e3 isolate was compatible with all hosts included in this bioassay, but the aggressiveness against different hosts varied as shown by the variation in DSI. Definitions of susceptible and resistant interaction seem to vary between different studies. Some *et al.* (1996) used DSI=25 and Strelkov *et al.* (2006 and 2007) used DSI=50 as cut-off value between susceptible and resistant interactions. Toxopeus *et al.* (1986) suggested that plants with DSI<20 should be classified as resistant, and plants with 20<DSI<80 as intermediate and DSI>80 as susceptible. The interpretation of the pathotype classification depends on which model for pathotype classification is being used (Table 2).

Table 2. Pathotype of the *P. brassicae* e3 isolate determined by different combinations of the two different disease severity assessment scales and three different definitions of susceptibility and resistance previously applied for pathotype classification of *P. brassicae* isolates.

	Pathotypes determined according to different definitions of resistance and susceptibility		
Symptom assessment method	Cut-off value: DSI=50 (Strelkov *et al.* 2006 and 2007)	Cut-off values: DSI<20= resistant and DSI>80=susceptible (Toxopeus *et al.* 1986)	Cut-off value: DSI = 25 (Some´ *et al.* 1996)
Six-graderd scale Crête *et al*. (1963)	Pathotype: 31/31/30	ECD 05,06,07,08,09 and 10 = susceptible. All other = intermediate	Pathotype: 31/31/31
Four-graded scale Kuginuku *et al.* (1999) + Strelkov *et al.* (2006)	Pathotype: 31/31/30	ECD 5, 9 and 10 = susceptible. All other = intermediate	Pathotype: 31/31/31

The upper parts of the plants showed little differences between inoculated and control plants. The amount of inoculum affects the symptom development (Hwang *et al.* 2011) and reducing the amount might have resulted in clearer differences between more and less susceptible hosts. Another source of variation between studies is, as with all bioassays, depending on visual assessment of disease severity, the human factor.

ECD 11, cv. Badger Shipper, had the lowest DSI and holds greatest prospects of being used as a partly resistant control for further studies on the e3 isolate. ECD 11 was, however, not included in the bioassay conducted by Fähling *et al.* (2003). The standard deviation of DSI was high for ECD 11, as for most of the hosts, implying inconsistent disease development between individual plants.

The pathotype of the *P. brassicae* e3 isolate has been previously classified (Fähling *et al.* 2003, 2004) by using two different bioassays which included the ECD hosts 01, 02, 03, 04, 05, 06 and 15. Fähling *et al.* (2003) reported ECD hosts 01, 03 and 04 to produce incompatible reactions with the e3 isolate. In the later study the same authors reported the DSI for ECD hosts 03, 05 and 06 as 45, 97 and 86, respectively. Comparing results to other studies is complicated as a wide range of methods and definitions of susceptibility vs. resistance have been used.

To summarise, the results conducted on the pathotype of the e3 isolate show equal general trends with the turnips being less susceptible compared to cv. Granaat and *B. napus*. The level of DSI of the hosts corresponds to that of previous studies but the intermutual rating of resistance among the turnips (ECD 01-04) is contradicting between the three different studies regardless of which test method and interpretation guidelines being applied. Problems with inconsistent results are commonly associated with pathotype classification of *P. brassicae* isolates (Strelkov *et al.* 2007). Growth conditions and host and pathogen heterogeneity are two commonly identified sources of variation resulting in low comparability between different studies. Adding more plant genotypes to the test, for example those used by Hatakeyama *et al.* (2004) or commercial CR varieties would provide more information on the

host range of the e3 isolate and might enable identification of more effective CR genes against the e3 isolate. In conclusion, e3 isolate is a broad range aggressive single spore isolate with a perceptual preference of cv. Granaat and *B. napus*. It was compatible with all 15 ECD hosts.

3. Optimization of cultivation system of test plants for gene expression analysis

3.1 Introduction

Biotrophic plant pathogens often depend on establishing intricate relationships with their hosts in order to access plant nutrients and overcome plant defence mechanisms without killing the host before the pathogen lifecycle is completed (Chandran *et al.* 2010, Hok *et al.* 2010). Establishing these intricate relationships involves alteration of biochemistry, metabolic patterns and physiology within the pathogen-host complex (Feng *et al.* 2012). Understanding the underlying processes and control mechanisms causing these changes in the *P. brassicae* host interaction will surely help in the development of new techniques to control clubroot. Genes with varying expression levels correlating to specific disease development stages have been postulated to be involved in the pathogenicity of *P. brassicae* (Ito *et al.* 1999, Brodman *et al.* 2002, Ando *et al.* 2006, Siemens *et al.* 2009). In order to study the processes involved in early life-stages such as resting spore germination, primary zoospore formation and formation of primary plasmodia a small manageable natural system with consistent infection and pathogen development rates is needed. Therefore, a small-scale system is tested for molecular-based investigations of the early infection stages in the *P. brassicae*/host interactions.

The RNA samples are used for gene expression analysis, which is a widely used tool in biological research for acquiring important information on genes involved in plant-pathogen interactions.

When using gene-expression analysis, several parameters need to be controlled. This includes amount of starting material, enzymatic efficiencies and differences between tissues in overall transcriptional activity (Vandesompele *et al.* 2002). Ideally the gene transcript number would be standardized to the number of cells. This is not possible when starting with solid tissues. Instead, internal control genes are most frequently used to normalize the mRNA fraction. The β-actin gene is commonly used as a universal reference gene to normalize qPCR data (Zyzynska-Granica and Koziak 2012). The expression of the control gene should ideally not vary depending on host tissue, duration of the experiment or experimental treatment. Considerable variation of expression of actin genes has been shown in other tissues (Zyzynska-Granica and Koziak 2012, Henn *et al.* 2013).

Genomic DNA can also be used for normalisation of gene expression analyses (Huggett *et al.* 2005). The main problem using DNA quantification as normalization method in general is that proliferating cells in general have variable haplotypes, resulting in varying numbers of sets of genetic information relative to the number of cells, compared to non-proliferating cells. This difference is usually less than two-fold in eukaryotic organisms. However, *P. brassice* could possibly constitute an exception since its lifecycle includes haploid and diploid mono- and multinuclear plasmodia (Tommerup and Ingram 1971, Kageyama and Asano 2009).

Some potentially interesting genes of *P. brassicae*, with putative functions in the infection cycle, were selected from the genome sequence draft or literature.

The *P. brassicae* genome draft (Schwelm *et al.* unpublished) contains a gene encoding a protein with homologies to cytokinin oxidases. Cytokinin oxidases play a major role in regulating cytokinin levels in plants (Avalbaev *et al.* 2012). Cytokinin levels deviate from normal levels in host tissues upon *P. brassicae* infection (Dekhuijzen 1981, Müller and Hilgenberg 1986, Devos *et al.* 2005, 2006) and presumably play an important role for the disease development (Siemens *et al.* 2006). There is evidence that cytokinin can be synthesized by the plasmodia (Dekhuijzen 1981, Müller and Hilgenberg, 1986), but alternatively the plasmodia can also function as cytokinin sink in some stages of infection (Devos *et al.* 2005).

Another important plant hormone altered by *P. brassicae* infection is auxin (Rauch *et al.* 1983, Devos *et al*. 2005, 2006). The production of the auxin indole 3-acetic acid (IAA) can be stimulated by *P. brassicae* but the actual auxin production is presumed to be carried out by host tissues (Grsic *et al.* 1999, Grsic-Rausch *et al.* 2000), and the secondary plasmodia function as auxin sinks (Devos *et al.* 2005). One study showed that up-regulation of auxin is transient in susceptible hosts but not in resistant hosts (Ludwig-Müller *et al.* 2009). Auxins are involved in many plant growth and developmental processes including remodelling of the cell wall so that cellulose microfibrils can move apart or slide past each other stretching out the cell wall, a prerequisite for hyperthrophic cell growth (Fry 1995, Cosgrove 2000). A putative auxin-responsive gene belonging to the GH3 family of auxin-responsive genes, known to be involved in in the regulatory network of plant growth and development processes (Abel and Theologist 1996, Terol *et al.* 2006), has been identified in the *P. brassicae* genome (Schwelm *et al.* unpublished).

A methyltransferase possibly involved in salicylic acid (SA) metabolism has been found in the *P. brassicae* genome sequence draft. SA is a key signalling molecule in inducible plant defence against biotrophic pathogens (Bari and Jones 2009, Pieterse *et al.* 2009) presumably including *P. brassicae* (Lovelock *et al.* 2013). Suppression of plant defence responses is vital for pathogen proliferation (Park *et al.* 2007).

A *P. brassicae* serine protease gene, *Pro-1*, has been found (Feng *et al.* 2010). Gene expression of *Pro-1* has been detected between four and 21 DPI and the expression was up-regulated compared to actin. The gene product was demonstrated to stimulate resting spore germination when mixed with root exudates secreted from plants. Serine proteases are enzymes cleaving peptide bonds in proteins and have been identified as pathogenicity factors in other pathosystems (Redman and Rodriguez 2002), most likely also in *P. brassicae.*

The last gene chosen for investigation encodes a trehalose-6-phosphatesynthase (Brodmann *et al.* 2002) which synthesizes trehalose (Müller *et al.* 1995). The disaccharide trehalose (α-D-glucopyranosyl1-[1,1]-α-D-glucopyranoside) is involved in carbon sink initiation and/or maintenance. Trehalose levels can be up to 20 times higher in infected root tissues compared to healthy cabbage plants (Keen and Williams 1969). Trehalose has a well-documented ability to protect enzymes and membranes and many organisms accumulate trehalose under stress conditions or during interactions with microbes (Müller *et al.* 1995). Trehalose accumulation is believed to affect the plant carbon metabolism both by interfering with the plant's sugar-

sensing system and by diversion of carbon away from plant metabolism, thus creating a carbon sink. Brodmann *et al.* (2002) hypothesized that trehalose was mainly synthesized by *P. brassicae* plasmodia and released into the host plant.

Aim

The aim of this experiment was to develop, test and evaluate methods to study the gene expression of selected genes of *P. brassicae* in resting spores, germinating zoospores and early infection stages in soil and plant roots synchronously.

Hypothesis

- The one ml pipette tip cultivation system can be used as a convenient growing system from which *P. brassicae* mRNA can be extracted from soil and from plant roots using commercial kits.
- Interactions with different host plants will result in different gene expression patterns depending on susceptibility of the host.
- The ratio between *P. brassicae* DNA and plant DNA will increase as the disease establishes and spreads within the plant roots.

3.2 Materials and methods

Cultivation, sampling and inoculation of test plants

A small-scale infection system was set up and tested to investigate early interactions between *P. brassicae* and hosts. One ml pipette tips were filled with growth matrix containing a 50:50 (volume) mix of sand and Hasselfors garden S-soil as reduced root growth was observed using pure sand.

Seeds of cv. Express, cv. Granaat and cv. Mendel were surface sterilised by dipping in 70% ethanol for 30 sec followed by rinsing in autoclaved water and incubation in sodium hyperchloride solution (1% active chlorine) containing a few drops of TWEEN-20 for 20 min. The surface sterilised seeds were pre-germinated for four days on moist filter paper at room temperature. Germinated seedlings were pushed down in the growth matrix of the pipette tips which were placed inside a Pipetman box (Gilson) filled with sterile 0.1% Blomstra (Cederroth International AB) (pH 6.5) (Fig. 5) and grown in a growth chamber set at a 22/16°C and 16/8h day/night cycle.

Half of the plants were inoculated with $2 \cdot 10^6$ *P. brassicae* resting spores per plant and the rest of the plants were mock-inoculated with autoclaved water. After inoculation the inoculated and mock-inoculated plants were housed in separate boxes. The plants were grown in a growth chamber set at 22/16°C and 16/8h day/night cycle. Seeds of cv. Express did not germinate evenly and were discarded. Root and soil samples were collected from the pipette tips sown with seeds of cv. Granaat or cv. Mendel. Three pipette tips from each box were collected at 2, 3, 4, 5 and 6 DPI and two pipette tips from each box were collected at 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 DPI. The contents of the tips were blown out of the tips using a Pasteur pipette. Two or three plants taken from the same box where pooled and constitute one sample. The soil was removed from the roots and collected in a 15 ml Falcon tube. The soil

samples were immediately frozen in liquid nitrogen and stored at -70°C until further use. The roots were thoroughly rinsed in tap water. The plants were cut off at the base of the stem and the roots were collected in 2.2 ml sterile microcentrifuge tubes, immediately frozen in liquid nitrogen and stored at -70°C until being ground into a fine powder using a mortar and pestle. The ground samples were divided into two equal halves and transferred into two 1.5 ml microcentrifuge tubes. The tubes were immediately frozen in liquid nitrogen and stored at -70°C until further use.

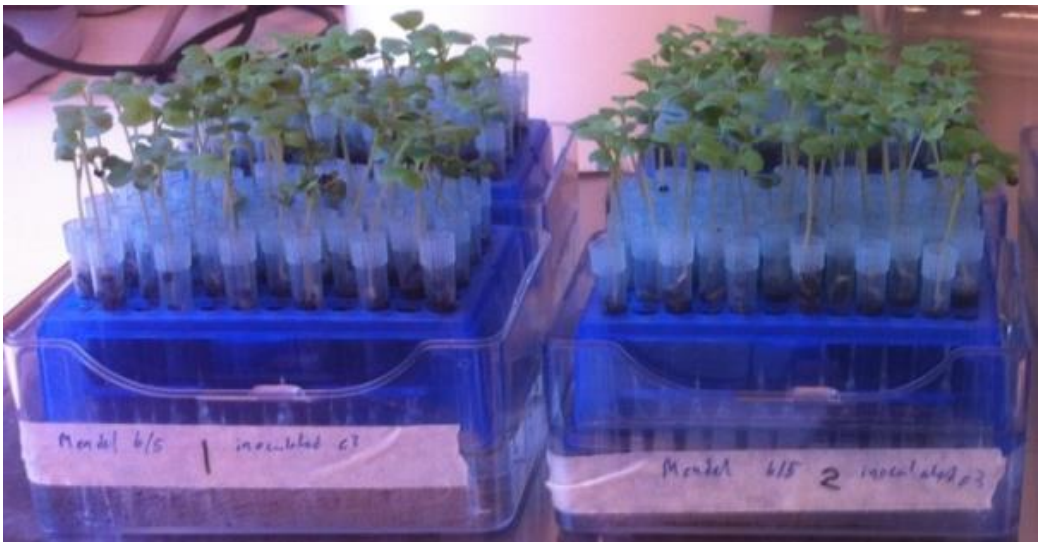

Fig. 5. Cultivation of cv. Mendel in Gilson Pipetman boxes filled with one ml pipette tips and nutrient solution at two DPI by the *P. brassicae* e3 isolate.

RNA extraction from soil samples

The soil samples were freeze-dried and DNA and RNA were extracted from the soil using RNA PowerSoil® Total RNA Isolation Kit (MoBio) together with RNA PowerSoil® DNA Elution Accessory Kit according to the manufacturer's protocol. After extraction of DNA and RNA the concentrations were measured in the samples using a NanoDrop spectrometer.

Preparation of RNA from root samples

RNA was extracted from the ground root samples using Spectrum™ Plant Total RNA Kit (protocol A) (Sigma). The samples were DNase treated with the TURBO DNA-free™ Kit (Ambion) according to the manufacturer's instructions. The RNA concentrations were measured with QUBIT and adjusted to the concentration of the lowest sample (15 ng/µl) by adding nuclease free water. A total of 15 µl of the RNA solution was transcribed into cDNA using the qScript RT (Quanta Biosciences) according to the manufacturer's instructions (mixed with 4 µl of qScript Reaction Mix (5X) and 1 µl qScript RT (Quanta Biosciences) mixed by vortexing and placed inside a thermal cycler programmed at 22°C for 5 min, 42°C for 30 min and 85°C for 5 min). The samples were kept at 4°C until stored at -20°C.

qPCR gene expression analysis

Six primer pairs (Appendix 1) were selected to test *P. brassicae* gene expression. Primer pairs Pbr002, Pbr003, Pbr018 for the actin, pro-1 and trehalose-P-synthase genes, respectively, have been described by Feng *et al.* (2013). The PBSMT-1 primer pair for the

methyltransferase gene was obtained from Ludwig-Müller (TU Dresden, Germany). Primer pairs for the putative GH3/ CytOx were designed using Primer3 software (http://frodo.wi.mit.edu/) and the *P. brassicae* genome sequence data.

Dilution series to evaluate primer effectiveness were produced using PCR amplified cDNA. The cDNA was amplified using the same primer pairs that were to be used later in the qPCR. The amplified PCR products were loaded on a 0.1% agarose gel, isolated, eluted and purified using GeneJET® Gel Extraction Kit (Thermo Scientific). The DNA concentration was measured using a QUBIT® 2.0 Fluorometer and adjusted to 0.0424 ng/µl. A dilution series including 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6-fold dilutions were prepared from the PCR products of each primer pair.

The wells of 96-well qPCR plates were filled with 5 µl template (15 ng cDNA/µl), 12.5 µl (2X) DyNamo Flash Sybr Green qPCR mix (Thermo Scientific), 1.5 µl 5mM forward primer, 1.5 µl 5 mM reverse primer and 4.5 µl nuclease free water. The wells were sealed with qPCR sealing film and placed inside the qPCR machine. Three different qPCR programs were initially tested with all six primer pairs (Appendix 1). Standard curves, amplification-peak-charts and amplification charts were generated by the qPCR software, BioRad IQ5. E-values were calculated using the formula:

$$Log(E) = \frac{-1}{Slope} \times \mathrm{Log}(10) - Log(1)$$

The results were evaluated and it was concluded that the primers are working the best when using qPCR program 3 (Appendix 1) as there was no unspecific amplification and the efficiency of the PCR for all primer pairs was close to 2.0. qPCR program 3 was therefore used for all the following qPCRs. The cDNA samples were loaded in 96-well PCR plates together with master mix and primers as described earlier. Each 96-well PCR plate was loaded with two replicates of the 36 cDNA samples, dilution series and one non-template control.

Analysis of qPCR gene expression results

Standard curves were generated from the dilution series and E-values were calculated for each primer pair (Invitrogen 2013). Melting curves and PCR amplification charts were generated and evaluated. The initial analysis showed that the results were not reliable and no further analyses were conducted.

Preparation and analysis of DNA from root samples

DNA was extracted from the ground root samples using Qiagen DNeasy Plant Mini Kit following the manufacturer's instructions. The DNA samples were stored at -24°C. *P. brassicae* and plant DNA contents were analysed by Eurofins Food and Agro Testing Sweden AB according to their standard assay using general primers for the plant cytochrome oxidase (COX) and *P. brassicae* ribosomal DNA as earlier described (Wallenhammar *et al.* 2012).

3.3 Results

RNA extraction from infected soil

After absorbance measurement of the RNA samples with a NanoDrop spectrometer, it was clear that the quantity and quality of extracted RNA were too low to study the gene expression of *P. brassicae* genes using RNA extracted from soil samples.

DNA content in infected roots

The ratio between *P. brassicae* and plant DNA (Pb/Cox gene ratio) in the samples was consistently higher in samples from *P. brassicae* inoculated root samples compared to the negative control (Fig. 6). There was no significant interaction between the plant and *P. brassicae* DNA ratio and time post infection (R^2=0.016) in inoculated plants.

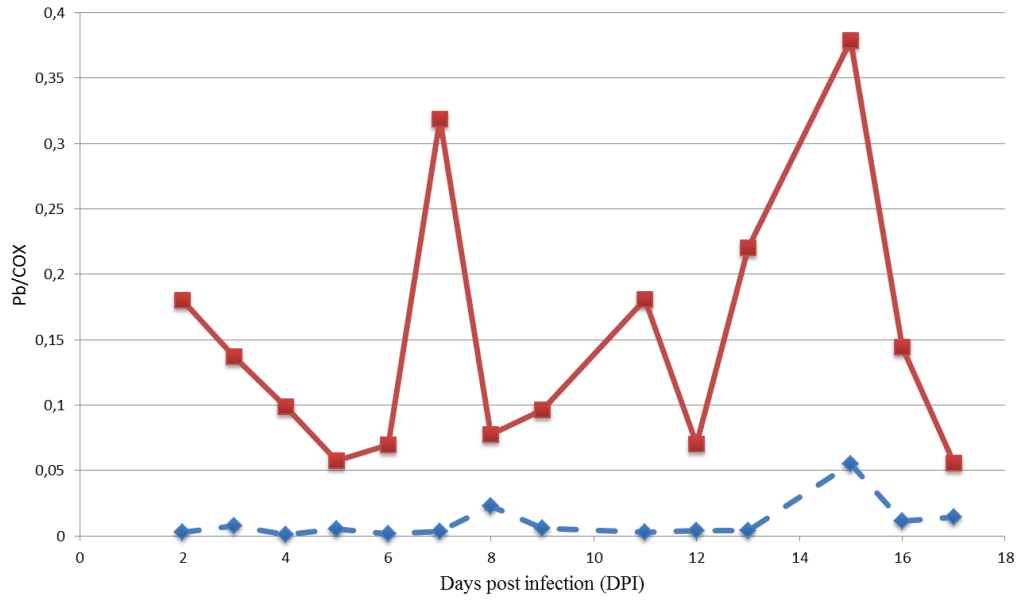

Fig. 6.. Average Pb/Cox gene ratio of inoculated (—■—) and non-inoculated (···◆···) *Brassica rapa* var. *chinensis* cv. Granaat samples at different time-points (DPI). There was no significant interaction between PB/COX ratio and DPI in inoculated plants (R^2=0.016).

Gene expression of *P. brassicae* in infected roots

The primer pairs CytOX and GH3 amplified unspecific PCR products and were not used in further experiments. Standard curves for the four other primer pairs Pbr002, Pbr003, Pbr018 and PBSMT-1 (Fig. 7) had E-values ranging between 2.226 and 2.016 and R2 values ranging from 0.916 to 0.996 (Table 3). The melt-peak charts of the 4 working primers (Fig. 8) displayed evenly distributed melt curves with only one distinct peak for each primer pair indicating that there was no amplification of unspecific DNA fragments or primer-dimers formed during the qPCR.

Table 3. E-values and correlation of the standard curves of primer pair Pbr002, Pbr 003, Pbr 018 and PBSMT.

	Pbr002	Pbr003	Pbr018	PBSMT
E	2.016	2.188	2.226	2.017
R^2	0.996	0.954	0.952	0.916

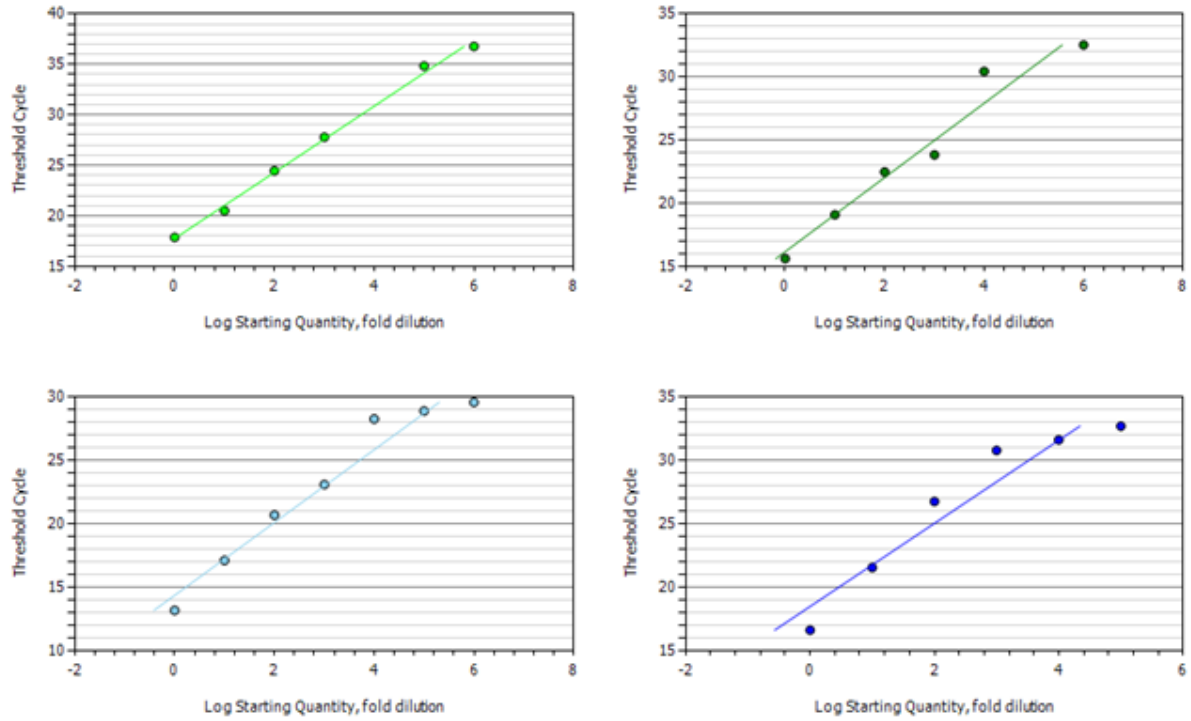

Fig. 7. Standard curves of qPCR products of the dilution series containing 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 fold diluted template concentrations of PCR-amplified cDNA with the primer pairs Pbr002 (upper left graph), Pbr003 (upper right graph), Pbr018 (lower left graph) and PBSMT-1 (lower right graph).

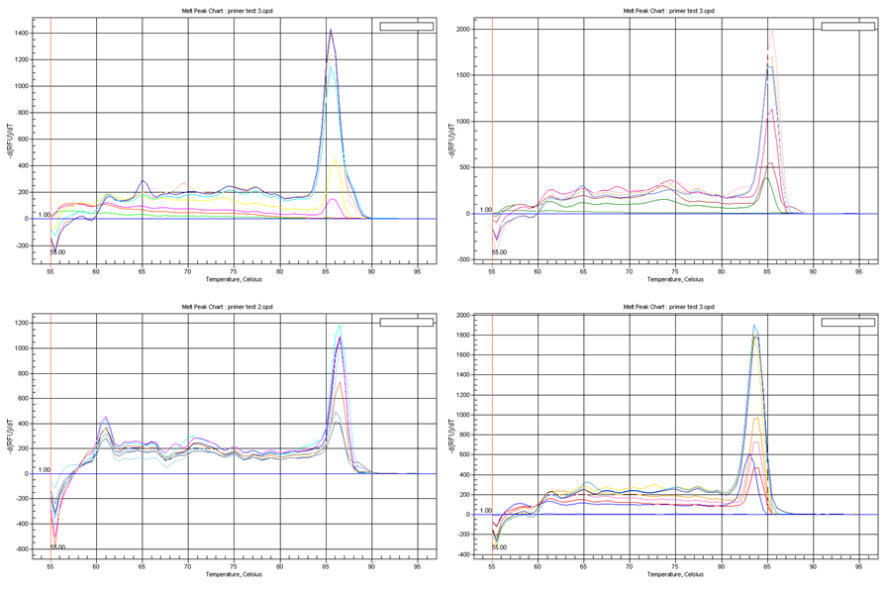

Fig. 8 Melt peak charts of qPCR products of the dilution series containing 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 fold diluted template concentrations with the primer pairs Pbr002 (upper left graph), Pbr003 (upper right graph), Pbr018 (lower left graph) and PBSMT-1 (lower right graph).

When running the qPCR gene expression analysis on the cDNA samples from inoculated and non-inoculated cv. Granaat roots under the same conditions as the dilutions series had been

tested, E-values and correlation of the dilution series deteriorated, melt peak charts displayed different patterns and there was amplification in non-template controls (Appendix 2).

3.4 Discussion

The cultivation system using one ml pipette tips can provide a simple and clean system for growing plants to study *P. brassicae*-host interactions. The sampling of both root and soil samples could be done easily and a consistent amount of soil from the rhizosphere could be sampled. However, the infection process did not appear to occur simultaneously on each plant, but rather showed a high variation based on the PB/COX ratio. Including more plants in each sample would reduce the variation of the sample caused by the variation between individual pipette tips. This would also have made the DNA and RNA extractions easier since the amount of plant tissue would have been increased. Adding soil from more samples would not have provided any benefits for the soil RNA extraction since one pipette tip contains about the maximum amount of soil allowed for the RNA extraction kit used. Studying gene expression in *P. brassicae* at single life-stages is not possible using this system since infections are likely to occur at different time-points and the roots will therefore simultaneously contain several life stages of the pathogen. Developing a system where inoculation can be controlled so that infections could only occur during a limited timeframe would provide better opportunities to study gene expression during distinct processes in pathogen development to identify transition stages in the pathogen lifecycle resulting in pivotal changes in the plant-pathogen complex.

Four out of the six tested primer sets were functional in the PCR program used in this study. The PCR program used generated good PCR efficiencies (E-values) for these four primers and could be used for further studies on *P. brassicae* gene expression.

The qPCRs performed did not generate reliable results because of amplification in non-template controls. Hence, the results were not further analysed. The dilution series had lower E-values when running the qPCR plates containing the samples and amplification was detected in non-template controls. This is most likely caused by the human factor, pipetting mistakes and exposure of the Sybr-green dye to light. The qPCR results might have been improved by higher cDNA quality and concentrations. Larger samples including more plants should have been taken to compensate for uneven disease development.

4. Stimulating effect on resting spore germination by root exudates of inoculated plant roots

4.1 Introduction

Germination of dormant *P. brassicae* resting spores occurs spontaneously at low rates but can be stimulated by various biotic and abiotic factors in its environment (Friberg *et al.* 2005, Dixon 2009b). Presence of host plants is presumed to stimulate resting spore germination. Using bait crops to stimulate resting spore germination in infested soils, hence eroding the resting spore population, is one of two major methods attempted for biocontrol of *P. brassicae* (Friberg *et al.* 2006, Donald and Porter 2009). Susceptible cabbages have shown the greatest effect on resting spore population decline (Dixon 2009b) and the use of susceptible crops as bait crops has been reported to reduce disease severity significantly in following Brassica crops (Harling and Kennedy 1991). However, the effect has been shown to be inconsistent (Friberg *et al.* 2006, Ahmed *et al.* 2011) and associated with the risk of pathogen multiplication (Robak 1996). Using clubroot resistant varieties can also decrease resting spore populations in soils (Hwang *et al.* 2013). Resting spore germination might be triggered by non-host species (Kowalski and Bochow 1996, Friberg *et al.* 2005).

In aquatic laboratory systems, root exudates of some plant species can stimulate resting spore germination (Friberg *et al.* 2006, Feng *et al.* 2010). The exact nature of the stimulating substance is not known, but Suzuki *et al.* (1992) concluded it to be to be a polar, heat stable, low molecular weight chemical secreted by plant roots. More recently a serine protease encoded by the *P. brassicae Pro-1* gene capable of increasing resting spore germination has been described (Feng *et al.* 2010). However, *Pro-1* only had an effect on resting spore germination in combination with plant root exudates.

Microbial communication (quorum sensing) is a well-known pathogenicity factor for several prokaryotic and some eukaryotic plant pathogens helping microorganisms to coordinate growth and development and efforts in breaking down host defence (Hornby *et al.* 2001, Albuquerque and Canadevall 2012).

Aim

The aim of this experiment was to conduct a pilot study to investigate whether root exudates of infected plants with different stages of *P. brassicae* infections can affect resting spore germination/dormancy compared to root exudates of healthy plants or not.

Hypothesis

Plant roots infected with *P. brassicae* secrete germination-stimulating factors during early infection stages.

4.2 Materials and methods

Root exudate solutions were obtained from *B. rapa* cv. Granaat. Seeds were surface sterilised as described in Chapter 3.2, germinated for two days and planted in autoclaved one ml pipette tips filled with autoclaved growth matrix consisting of 50:50 (volume) mix of sand and Hasselfors garden S-soil. The pipette tips were placed inside an autoclaved Pipetman box

(Gilson) filled with sterile 0.1% Blomstra (Cederroth International AB) (pH 6.5) and the plants were grown in a growth chamber set at a 22/16°C and 16/8h day/night cycle.

The plants in the four boxes were kept separated and plants were inoculated at three different time-points: eleven, nine and five days before the plants were being harvested. The plants in the fourth box were not inoculated and served as control. The inoculum consisted of $2 \cdot 10^6$ cleaned e3 resting spores re-suspended in 200 µl sterile water per plant. The plants were harvested 15 days post planting by blowing them out of the pipette tips using a Pasteur pipette. For producing the root exudate solutions, roots of 10 plants were rinsed in autoclaved water, cut off and incubated in 7 ml 0.1 M MOPS buffer (pH 6.2) in 15 ml Falcon tubes wrapped in tinfoil. One Falcon tube containing only 0.1 M MOPS buffer and one Falcon tube containing only sterile water were also wrapped in tinfoil. The Falcon tubes were incubated in a growth chamber set at 22/16°C, 16/8 h day/night cycle (Feng *et al.* 2010). After 36 h the samples were centrifuged at 600 rpm and the supernatant was filter-sterilised using a 0.22 µm pore filter.

Resting spores were treated with root exudates by mixing 800 µl root exudate solution and 400 µl of $4.2 \cdot 10^7$ spores/ml spore suspension in 1.5 ml microcentrifuge tubes wrapped in tinfoil and incubated at 28°C. At 0, 2, 4, 6, 8, 10, 13 and 19 DPI the spore germination was determined microscopically. To prepare microscope samples, 10 µl aliquots of the root exudate/spore solution were air-dried on a microscope slide for 1 hr and subsequently stained with 1% Orcein by adding 10 µl of staining solution onto the dried resting spores and carefully flaming the slide for approximately two seconds (Naiki *et al.* 1987). The Orcein was air-dried for an hour and the samples were covered with 10 µl sterile water and a cover slip. The ratio between germinated and ungerminated resting spores was determined by sweeping the microscope across an axis, counting 200 resting spores situated along the axis in each sample (Friberg *et al.* 2005).

Ungerminated resting spores are deeply stained and could be differentiated from unstained germinated spores with an empty appearance, often with visible exit pores from where the zoospores had been released (Fig. 9) (Naiki *et al.* 1987). For each time point between 4 and 12 samples were investigated.

A simple linear regression between the single predictor variable (time) and the response variable (resting spore germination frequency) of each treatment was calculated and the correlation coefficient R^2 was calculated in Microsoft Office Excel 2010.

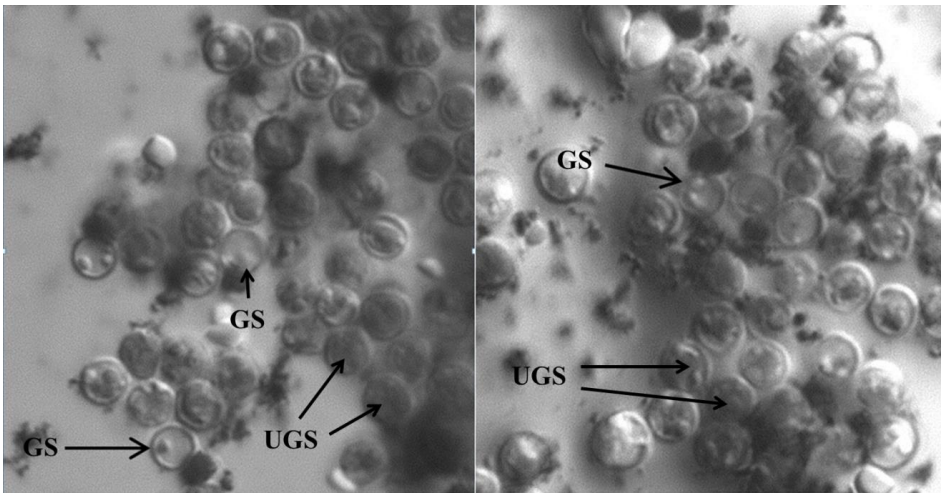

Fig. 9. Ungerminated (UGS) and germinated (GS) resting spores of *P. brassicae* incubated in root exudates of healthy plants for two days (left) and root exudates of plants harvested at five DPI (right).

3.3 Results

An interesting observation was that the colours of the root exudate solutions were more yellowish the longer the roots had been exposed to *P. brassicae* (Fig. 10). The colour of the root exudate solution of the non-inoculated controls could not be distinguished from the colour of the MOPS buffer.

A higher germination rate was observed in the samples with root exudates from inoculated roots compared to root exudates of non-inoculated plants (Fig. 11). After 19 days of incubation, the frequency of germinated resting spores were 63% for spores incubated in root exudate solution from plants exposed to *P. brassicae* for five days, 63% for spores incubated in root exudate solution from plants exposed to *P. brassicae* for nine days, 55% for spores incubated in root exudate solution from plants exposed to *P. brassicae* for eleven days, 52% for spores incubated in root exudate solution from non-inoculated plants and 26% for spores incubated in MOPS buffer. The resting spore germination rate of spores incubated in root exudate solution from non-inoculated plants was 1.9 times higher compared to the germination rate of spores incubated only in MOPS buffer (Table 4). The resting spore germination rates of spores incubated in root exudate from plants inoculated eleven, nine and five days before harvest were 2.0, 2.4 and 2.4 times higher, respectively, compared to the resting spore germination rate in only MOPS.

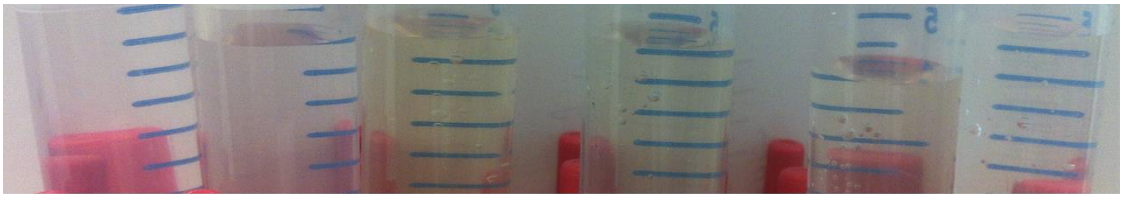

Fig. 10. Colours of root exudate solutions of cv. Granaat.

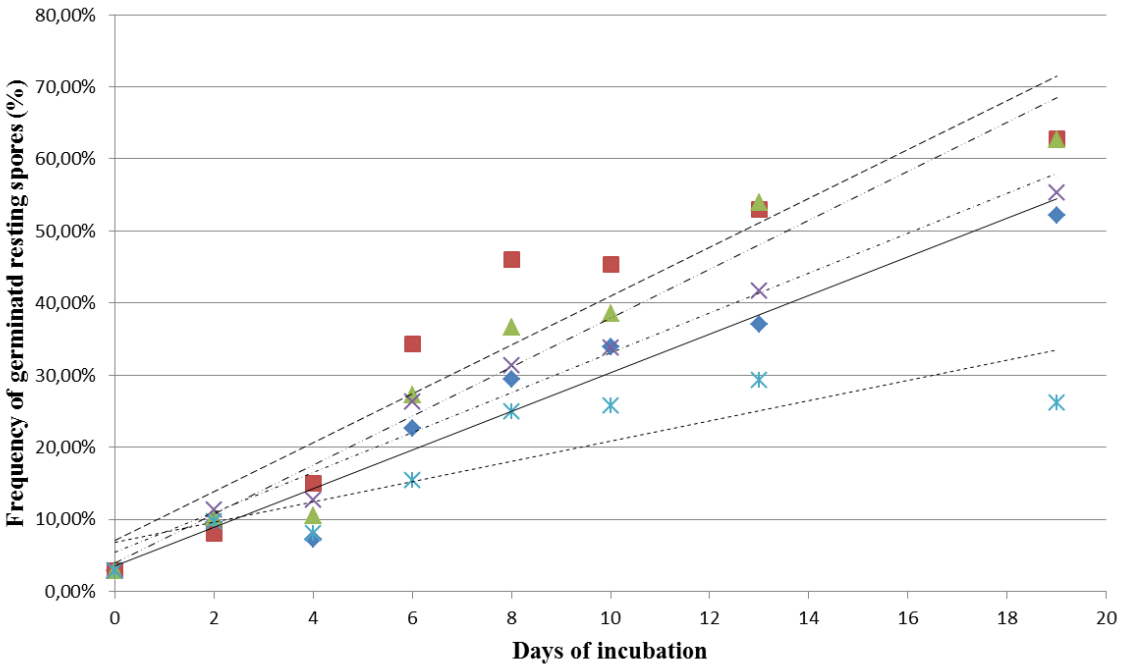

Fig. 11. Frequency (%) and linear regression lines for frequency of germinated *P. brassicae* resting spores after 0, 2, 4, 6, 8, 10, 13 or 19 days of incubation in MOPS buffer (pH 6.2) (), root exudate solution of non-inoculated plants (), and root exudate solution from plants exposed to *P. brassicae* for 5 (), 9 () and 11 () days.

Table 4. Resting spore germination rate, relative resting spore germination rate compared to the resting spore germination rate in MOPS buffer and R^2 values for the correlation between the frequency of germinated resting spores and incubation time.

Treatment	Resting spore germination rate (%/day)	Relative resting spore germination rate compared to MOPS buffer(pH 6.2)	R^2 value
Root exudate solution of plants exposed to *P. brassicae* for 5 days	3.39	2.42	0.89
Root exudate solution of plants exposed to *P. brassicae* for 9 days	3.40	2.43	0.95
Root exudate solution of plants exposed to *P. brassicae* for 11 days	2.76	1.97	0.97
Root exudate solution of non-inoculated plants	2.68	1.91	0.95
MOPS buffer (pH 6.2)	1.40	1	0.75

4.4 Discussion

Root exudates appear to stimulate resting spore germination compared to MOPS buffer alone. The root exudates of plants exposed to *P. brassicae* for five and nine days caused 1.27 times greater resting spore germination stimulating effect compared to non-inoculated root exudates. Root exudates from plants exposed to *P. brassicae* for eleven days did not obviously increase resting spore germination rate compared to root exudates of non-inoculated plants. The frequency of germinated resting spores increases rapidly between four and eight days incubation. Hence using a linear model is likely to result in underestimation of the resting spore germination stimulating effect as the differences between treatments decreases at later time-points. The linear model was chosen to reduce presumed sampling and assessment errors and is estimated to be accurate enough to show differences between treatments. A more detailed analysis would have been enabled by replicating the experiment. The results can be interpreted as some early stages of a *P. brassicae* infections produce or increase production of germination-stimulants. Assuming a similar disease development pace as has been observed (Tommerup and Ingram 1971, Luo *et al.* 2013), the primary plasmodia would have started developing in the roots at 5 DPI and that the transition between primary and secondary infection stages would have taken place around eight to ten DPI. Therefore it could be assumed that resting spore germination is stimulated by root exudates produced during development of the primary plasmodia but not by the secondary plasmodia. This resting spore germination stimulation could be triggered by production of chemical substances by the pathogen, e.g. serine protease (Chapter 3), or pathogen-induced overproduction of resting spore stimulating chemicals by the host. The shifting colour of the root exudates apparently induced by *P. brassicae* inoculation shows that the chemical composition of the root exudates was being affected. The results imply that *P. brassicae* possesses a quorum sensing system, involved in controlling resting spore germination of dormant resting spores in the presence of susceptible hosts where primary plasmodia had been established. The effect presumably declines as *P. brassicae* infections transit between primary and secondary infection stages. Using quorum sensing to coordinate resting spore germination would presumably reduce the risk of a rapid decline in the resting spore population caused by presence of non-host plants, increasing the fitness of the pathogen, and could provide an explanation to why using non-host plants as bait crops has not been as successful as using susceptible brassica bait crops. Further investigations on this phenomenon should be undertaken and if the hypothesis that *P. brassicae* has evolved quorum sensing is true and the underlying mechanisms can be uncoiled the quorum sensing mechanisms could most likely be utilized in creating new efficient bait crops for control of *P. brassicae* and to identify chemicals stimulating resting spore germination.

The system used to create root exudates was simple and efficient to handle and it reduces the risk of contaminations. The concentration of resting spores in the root exudate solution was unnecessarily high as the spores were hard to separate from each other when assessing the frequency of resting spore germination. Determination of stages in *P. brassicae* infection development would have aided interpretation of the results.

5. General discussion

5.1 Concluding remarks

The results of the experiments conducted in this thesis could be condensed into four main results:

The e3 isolate was able to infect and cause symptoms on all 15 ECD hosts. The pathotype of the isolate depends on the method for disease assessment and calculation of DSI and how the border between susceptibility and resistance is defined.

The attempt to create a convenient, natural, reliable and efficient system to study *P. brassicae* gene expression using a cultivation system with one ml pipette tips was working suboptimally since the DNA analysis indicated significant variations in infection development between individual plants. However, the method was convenient to use for creating root exudates for studies on germination of resting spores.

The qPCR program and four of the primers tested can be used for further studies on the selected genes but the gene expression analysis in this thesis did not generate reliable results.

Resting spore germination is stimulated by root exudates of susceptible plants. Inoculated roots can likely stimulate resting spore germination further but the effect is seemingly dependent on the infection stage of *P. brassicae* in the roots. This could be interpreted as *P. brassicae* using quorum sensing to break resting spore dormancy in presence of susceptible plants.

5.2 Acknowledgements

I would like to thank my supervisor Dr Arne Schwelm and assistant supervisor Professor Christina Dixelius for all their support and inspiration.

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6. References

Abel, S. and Theologist, A. (1996) Early genes and auxin action. *Plant Physiol.* **111**, 9-17.

Agarwal, A., Kaul, V., Faggian, R., Rookes, J.E., Ludwig-Muller, J. and Cahill, D.M. (2011) Analysis of global host gene expression during the primary phase of the *Arabidopsis thaliana-Plasmodiophora brassicae* interaction. *Funct. Plant Biol.* **38**, 462-478.

Ahmed, H.U., Hwang, S.F., Strelkov, S.E., Gossen, B.D., Peng, G., Howard, R.J. and Turnbull, G.D. (2011) Assessment of bait crops to reduce inoculum of clubroot *(Plasmodiophora brassicae)* of canola. *Can. J. Plant Sci.* **91,** 545–51.

Albuquerque, P. and Casadevall, A. (2012) Quorum sensing in fungi - a review. *Med. Mycol.* **50**, 337-345.

Ando, S., Yamada, T., Asano, T., Kamachi, S., Tsushima, S., Hagio, T. and Tabei, Y. (2006) Molecular cloning of PbSTKL1 gene from *Plasmodiophora brassicae* expressed during club root development. *J. Phytopathol.* **154,** 185–189.

Asano, T., Kageyama, K. and Hyakumachi, M. (1999) Surface disinfestation of resting spores of *Plasmodiophora brassicae* used to infect hairy roots of *Brassica* spp. *Phytopathology.* **89,** 314-319.

Avalbaev, A.M., Somov, K.A., Yuldashev, R.A. and Shakirova, F.M. (2012) Cytokinin oxidase is key enzyme of cytokinin degradation. *Biochemistry-Moscow* **77**, 1354-1361.

Bari, R. and Jones, J. (2009) Role of plant hormones in plant defence responses. *Plant Mol. Biol.* **69**, 473-488.

Brodmann, D., Schuller, A., Ludwig-Muller, J., Aeschbacher, R.A., Wiemken, A., Boller, T. and Wingler, A. (2002) Induction of trehalase in *Arabidopsis* plants infected with the trehalose-producing pathogen *Plasmodiophora brassicae*. *Mol. Plant-Microbe Interact.* **15**, 693-700.

Buczacki, S.T., Toxopeus, H., Mattusch, P., Johnston, T.D., Dixon, G.R. and Hobolth, L.A. (1975) Study of physiologic specialization in *Plasmodiophora-Brassicae* - Proposals for attempted rationalization through an international approach. *Trans. Brit. Mycol. Soc.* **65**, 295-303.

Burki, F., Kudryavtsev, A., Matz, M.V., Aglyamova, G.V., Bulman, S., Fiers, M., Keeling, P.J. and Pawlowski, J. (2010) Evolution of Rhizaria: new insights from phylogenomic analysis of uncultivated protists. *Bmc Evol. Biol.* **10,** 377.

Chandran, D., Inada, N., Hather, G., Kleindt, C.K. and Wildermuth, M.C. (2010) Laser microdissection of *Arabidopsis* cells at the powdery mildew infection site reveals site-specific processes and regulators. *Proc. Natl. Acad. Sci. USA* **107**, 460-465.

Cosgrove, D.J. (2000) Loosening of plant cell walls by expansins. *Nature* **407**, 312–326.

Crête, R., Laliberte, J. and Jasmin, J.J. (1963) Chemical control of clubroot, *P. brassicae*, of crucifers in mineral and organic soils. *Can. J. Plant Sci* **43**, 349-354.

Crête, R. (1981) Worldwide importance of clubroot. *Clubroot Newslett.* **11,** 6–7

Dekhuijzen, M. (1981) The occurrence of free and bound cytokinins in plasmodia of *Plasmodiophora* brassica isolated from tissue cultures of clubroots. *Plant Cell Rep.* **1**, 18-20.

Deora, A., Gossen, B.D. and McDonald, M.R. (2012) Infection and development of *Plasmodiophora brassicae* in resistant and susceptible canola cultivars. *Can. J. Plant Pathol.-Rev. Can. Phytopathol.* **34**, 239-247.

Devos, S., Laukens, K., Deckers, P., Van der Straeten, D., Beeckman, T., Inze, D., Van Onckelen, H., Witters, E. and Prinsen, E. (2006) A hormone and proteome approach to picturing the initial metabolic events during *Plasmodiophora brassicae* infection on *Arabidopsis*. *Mol. Plant-Microbe Interact.* 19, 1431-1443.

Devos, S., Vissenberg, K., Verbelen, J.P. and Prinsen, E. (2005) Infection of Chinese cabbage by *Plasmodiophora brassicae* leads to a stimulation of plant growth: impacts on cell wall metabolism and hormone balance. *New Phytol.* **166**, 241-250.

Diederichsen, E., Frauen, M., Linders, E.G.A., Hatakeyama, K. and Hirai, M. (2009) Status and perspectives of clubroot resistance breeding in Crucifer crops. *J. Plant Growth Regul.* **28**, 265-281.

Dixon, G.R. (2009a) The occurrence and economic impact of *Plasmodiophora brassicae* and clubroot disease. *J. Plant Growth Regul.* **28**, 194-202.

Dixon, G.R. (2009b) *Plasmodiophora brassicae* in its environment. *J. Plant Growth Regul.* **28**, 212-228.

Donald, C. and Porter, I. (2009) Integrated control of clubroot. *J. Plant Growth Regul.* **28**, 289-303.

Fahling, M., Graf, H. and Siemens, J. (2003) Pathotype separation of *Plasmodiophora brassicae* by the host plant. *J. Phytopathol. Zeitschr*. **151**, 425-430.

Fahling, M., Graf, H. and Siemens, J. (2004) Characterization of a single-spore isolate population of *Plasmodiophora brassicae* resulting from a single club. *J. Phytopathol*. **152**, 438-444.

FAOSTAT (2013) Crops Area Harvested. http://faostat3.fao.org/home/index.html#DOWNLOAD: *Food and Agriculture Organization of the United Nations.*

Feng, J., Hwang, S.-F. and Strelkov, S.E. (2013) Assessment of gene expression profiles in primary and secondary zoospores of *Plasmodiophora brassicae* by dot blot and real-time PCR. *Microb. Res.* **168,** 518-524.

Feng, J., Hwang, R., Hwang, S.F., Strelkov, S.E., Gossen, B.D., Zhou, Q.X. and Peng, G. (2010) Molecular characterization of a serine protease Pro1 from *Plasmodiophora brassicae* that stimulates resting spore germination. *Mol. Plant Pathol.* **11**, 503-512.

Feng, J., Hwang, S.F. and Strelkov, S.E. (2012) Analysis of expressed sequence tags derived from a compatible *Plasmodiophora brassicae*-canola interaction. *Can. J. Plant Pathol.* **34**, 562-574.

Friberg, H., Lagerlof, J. and Ramert, B. (2005) Germination of *Plasmodiophora brassicae* resting spores stimulated by a non-host plant. *Eur. J. Plant Pathol.* **113**, 275-281.

Friberg, H., Lagerlof, J. and Ramert, B. (2006) Usefulness of nonhost plants in managing *Plasmodiophora brassicae*. *Plant Pathol*. **55**, 690-695.

Fry SC. (1995) Polysaccharide-modifying enzymes in the plant cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 497-520.

Grsic-Rausch, S., Kobelt, P., Siemens, J.M., Bischoff, M. and Ludwig-Muller, J. (2000) Expression and localization of nitrilase during symptom development of the clubroot disease in *Arabidopsis*. *Plant Physiol.* **122**, 369-378.

Grsic, S., Kirchheim, B., Pieper, K., Fritsch, M., Hilgenberg, W. and Ludwig-Muller, J. (1999) Induction of auxin biosynthetic enzymes by jasmonic acid and in clubroot diseased Chinese cabbage plants. *Physiol. Plant.* **105**, 521-531.

Hatakeyama, K., Fujimura, M., Ishida, M. and Suzuki, T. (2004) New classification method for *Plasmodiophora brassicae* field isolates in Japan based on resistance of F-1 cultivars of Chinese cabbage (*Brassica rapa L.*) to clubroot. *Breeding Sci.*, **54**, 197-201.

Harling R. and Kennedy SH. (1991) Biological control of *Plasmodiophora brassicae* using a bait crop. *Mededelingen – Faculteit Landbouwwetenschappen*, Rijksuniversiteit Gent **56**, 159–70.

Henn, D., Bandner-Risch, D., Perttunen, H., Schmied, W., Porras, C., Ceballos, F., Rodriguez-Losada, N. and Schafers, H.J. (2013) Identification of reference genes for Quantitative RT-PCR in Ascending Aortic Aneurysms. *Plos One*. **8**, 1-6.

Hok, S., Attard, A. and Keller, H. (2010) Getting the most from the host: How pathogens force plants to cooperate in disease. *Mol. Plant-Microbe Interact*. **23**, 1253-1259.

Horiuchi, S and Hori, M. (1980) A simple greenhouse technique for obtaining high levels of clubroot incidence. *Bull. Chugoku. Natl Agric. Exp. Stn. E.* **17,** 33–55.

Hornby, J.M., Jensen, E.C., Lisec, A.D., Tasto, J.J., Jahnke, B., Shoemaker, R., Dussault, P. and Nickerson, K.W. (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microb.* **67**, 2982-2992.

Howard, R.J., Strelkov, S.E. and Harding, M.W. (2010) Clubroot of cruciferous crops - new perspectives on an old disease. *Can. J. Plant Pathol.* **32**, 43-57.

Hugget, J., Dheda, K., Bustin, S. and Zumla, A. (2005) Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* **6**, 279-284.

Hwang, S.F., Ahmed, H.U., Zhou, Q., Rashid, A., Strelkov, S.E., Gossen, B.D., Peng, G. and Turnbull, G.D. (2013) Effect of susceptible and resistant canola plants on *Plasmodiophora brassicae* resting spore populations in the soil. *Plant Pathol.* **62**, 404-412.

Hwang, S.F., Ahmed, H.U., Zhou, Q., Strelkov, S.E., Gossen, B.D., Peng, G. and Turnbull, G.D. (2011) Influence of cultivar resistance and inoculum density on root hair infection of canola (*Brassica napus)* by *Plasmodiophora brassicae. Plant Pathol.* **60,** 820-829.

Hwang, S. F., Cao, T., Xiao, Q., Ahmed1, H. U., Manolii, V. P., Turnbull, G. D., Gossen, B. D., Peng, G. and Strelkov, S. E. (2012a) Effects of fungicide, seeding date and seedling age on clubroot severity, seedling emergence and yield of canola. *Can. J. Plant Sci.* **92,** 1175-1186

Hwang, S.F., Strelkov, S.E., Feng, J., Gossen, B.D. and Howard, R.J. (2012b) *Plasmodiophora brassicae*: a review of an emerging pathogen of the Canadian canola (*Brassica napus*) crop. *Mol. Plant Pathol.* **13**, 105-113.

Ingram, D.S. and Tommerup, I.C. (1972) Life-history of *Plasmodiophora-brassicae* Woron. *Proceed. Roy. Soc. B-Biol. Sci.* **180**, 103-112.

Invitrogen (2013) Important parameters of quantitative PCR (qPCR) analysis. https://tools.invitrogen.com/content/sfs/appendix/PCR_RTPCR/Important%20Parameters%20of%20qPCR.pdf.

Ito, S., Ichinose, H., Yanagi, C., Tanaka, S., Kameya-Iwaki, M., and Kishi, F. (1999) Identification of an in planta-induced mRNA of Plasmodiophora brassicae. *J. Phytopathol*. **147**, 79–82.

Jubault, M., Hamon, C., Gravot, A., Lariagon, C., Delourme, R., Bouchereau, A. and Manzanares-Dauleux, M.J. (2008) Differential regulation of root arginine catabolism and polyamine metabolism in clubroot-susceptible and partially resistant *Arabidopsis* genotypes. *Plant Physiol.* **146**, 2008-2019.

Kageyama, K. and Asano, T. (2009) Life cycle of *Plasmodiophora brassicae*. *J. Plant Growth Regul.* **28**, 203-211.

Keen, N. T., and Williams, P. H. (1969) Translocation of sugars into infected cabbage tissues during clubroot development. *Plant Physiol*. **44**, 748-754.

Kowalski, K. and Bochow, H. (1996) Observations on the behaviour of resting spores of *Plasmodiophora brassicae* in the presence of cruciferous and non-cruciferous plant roots. *Acta Horticulturae* **407,** 419–422.

Kuginuki Y, Hiroaki Y and Hirai M. (1999) Variation in virulence of *Plasmodiophora brassicae* in Japan tested with clubroot-resistant cultivars of Chinese cabbage (*Brassica rapa L*. spp. *pekinensis*). *Eur. J. Plant Pathol.* **105,** 327–332.

Lovelock, D.A., Donald, C.E., Conlan, X.A. and Cahill, D.M. (2013) Salicylic acid suppression of clubroot in broccoli (*Brassicae oleracea var. italica*) caused by the obligate biotroph *Plasmodiophora brassicae*. *Australasian Plant Pathol.* **42**, 141-153.

Ludwig-Müller, J., Prinsen, E., Rolfe, S.A. and Scholes, J.D. (2009) Metabolism and plant hormone action during clubroot disease. *J. Plant Growth Regul*. **28**, 229–244.

Luo, H-C., Chen, G-K., Liu, C-P., Huang, Y. and Xiao, C-G. (2013) An improved culture solution technique for *Plasmodiophora brassicae* infection and the dynamic infection in the root hair. *Australasian. Plant Pathol.* **42**, 1-8.

Matsumoto, E., Ueno, H., Aruga, D., Sakamoto, K. and Hayashida, N. (2012) Accumulation of three clubroot resistance genes through marker-assisted selection in Chinese cabbage *Brassica rapa* ssp. *pekinensis*. *J. Jpn. Soc. Hortic. Sci.* **8,** 184–190.

Müller, J., Boller, T. and Wiemken, A. (1995) Trehalose and trehalase in plants: recent developments. *Plant Sci*. **112**:1-9.

Müller, P. and Hilgenberg, W. (1986) Isomers of zeatin and zeatin riboside in clubroot tissue: evidence for trans-zeatin biosynthesis by *Plasmodiophora brassicae*. *Physiol. Plant.* **66**, 245–250.

Naiki, T., Dixon, G.R. and Ikegami, H. (1987) Quantitative estimation of spore germination of *Plasmodiophora-Brassicae*. *Trans. Brit. Mycol. Soc.* **89**, 569-572.

Niwa, R., Kawahara, A., Murakami, H., Tanaka, S. and Ezawa, T. (2011) Complete structure of nuclear rDNA of the obligate plant parasite *Plasmodiophora brassicae*: intraspecific polymorphism in the exon and group 1 intron of the large subunit rDNA. *Protist* **162,** 423-434

Oxley, S. (2007) Clubroot disease of oilseed rape and other Brassica crops. *Tech. Note Scott. Agr. Coll.* (TN602). ISBN: 1854828908.

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S. and Klessig, D.F. (2007) Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* **318,** 113–116

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S.C.M. (2009) Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* **5**, 308-316.

Rauch, T., Butcher, D.N. and Hilgenborg, W. (1983) Indole-3-methylgIucosinoIate biosynthesis and metabolism in clubroot diseased plants. *Physiol. Plant*. **58**, 93-100

Redman, R.S. and Rodriguez, R.J. (2002) Characterization and isolation of an extracellular serine protease from the tomato pathogen *Colletotrichum coccodes*, and its role in pathogenicity. *Mycol. Res.* **106**, 1427-1434.

Robak, J. (1996) Crop rotation effect on clubroot disease decrease. *Acta Horticulturae* **371**, 223–226.

Russell, J. and Bulman, S. (2005) The liverwort *Marchantia foliacea* forms a specialized symbiosis with arbuscular mycorrhizal fungi in the genus Glomus. *New Phytol.* **165**, 567-579.

Seskar, M., Shulaev, V. and Raskin, I. (1998) Endogenous methyl salicylate in pathogen-inoculated tobacco plants. *Plant Physiol.*, **116**, 387-392.

Siemens, J., Graf, H., Bulmans, S., In, O. and Ludwig-Müller, J. (2009) Monitoring expression of selected *Plasmodiophora brassicae* genes during clubroot development in *Arabidopsis thaliana*. *Plant Pathol.* **58,** 130-136.

Siemens, J., Keller, I., Sarx, J., Kunz, S., Schuller, A., Nagel, W., Schmulling, T., Parniske, M. and Ludwig-Muller, J. (2006) Transcriptome analysis of *Arabidopsis* clubroots indicate a key role for cytokinins in disease development. *Mol. Plant-Microbe Interact.* **19**, 480-494.

Some´, A., Manzanares, M.J., Laurens, F., Baron, F., Thomas, G. and Rouxel, F. (1996) Variation for virulence on *Brassica napus L. amongst Plasmodiophora brassicae* collections from France and derived single-spore isolates. *Plant Pathol.* **45,** 432–439.

Strelkov S.E., Tewari J.P. and Smith-Degenhardt E. (2006) Characterization of *Plasmodiophora brassicae* populations from Alberta, Canada. *Can. J. Plant Pathol.* **28,** 467–474.

Strelkov, S.E., Manolii, V.P., Cao, T., Xue, S. and Hwang, S.F. (2007) Pathotype classification of *Plasmodiophora brassicae* and its occurrence in *Brassica napus* in Alberta, Canada. *J. Phytopathol.* **155**, 706-712.

Suzuki, K., Matsumiya, E., Ueno, Y. and Mizutani, J. (1992) Some properties of germination-stimulating factor from plants for resting spores of *Plasmodiophora brassicae*. *Ann. Phytopathol. Soc. Jpn.* **58,** 699–705.

Swedish Board of Agriculture (2013) Åkerarealens användning efter län/riket och gröda. År 1981-2012. http://statistik.sjv.se/Database/ Jordbruksverket/Markanvandning/Markanvandning.asp: *Swedish Board of Agriculture.*

Terol, J., Domingo, C. and Talon, M. (2006) The GH3 family in plants: Genome wide analysis in rice and evolutionary history based on EST analysis. *Gene* **371**, 279-290.

Tingal, H. and Webster J. (1981) Technique for single spore infection by *Plasmodiophora brassicae*. *Trans. Br. Mycol. Soc.* **76,** 187-190.

Tommerup, I.C. and Ingram, D.S. (1971) Life-cycle of *Plasmodiophora-Brassicae* Woron. in Brassica tissue cultures and in intact roots. *New Phytol.* **70**, 327-332.

Toxopeus, H., Dixon, G.R. and Mattusch, P. (1986) Physiological specialization in *Plasmodiophora brassicae*: an analysis by international experimentation. *Trans. Br. Mycol. Soc*. **87,** 279–287.

Wallenhammar, A.C. (1996) Prevalence of *Plasmodiophora brassicae* in a spring oilseed rape growing area in central Sweden and factors influencing soil infestation levels. *Plant Pathol.* **45**, 710-719.

Wallenhammar, A.C., Johnsson, L. and Gerhardson, B. (2000) Agronomic performance of partly clubroot-resistant spring oilseed turnip rape lines. *Phytopath. Z.*. **148**, 495-499.

Wallenhammar, A-C., Almquist, C. and Jonsson, A. (2012) In-field distribution of *Plasmodiophora brassicae* measured using quantitative real-time PCR. *Plant Pathol.* ***6*1,** 16-28

Werner, S., Diederichsen, E., Frauen, M., Schondelmaier, J. and Jung, C. (2008) Genetic mapping of clubroot resistance genes in oilseed rape. *Theor. Appl. Genet.* **116**, 363-372.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. **3**, 1-11

Voorrips, R.E. (1996) Production, characterization and interaction of single-spore isolates of *Plasmodiophora brassicae*. *Eur. J. Plant Pathol.* **102**, 377-383.

Zyzynska-Granica, B. and Koziak, K. (2012) Identification of suitable reference genes for real-time PCR analysis of statin-treated human umbilical vein endothelial cells. *Plos One* **7**, 1-12.

Appendix 1

Primer sequences and PCR program

Table 1. Primer sequences and properties.

ID	Accession	Plasmo0.3	name	FWD	REV	bp	Ref
Pbr002	ACY27467		Pro-1	CAAACGTGGTGTTCACGAAT	GTGCAGGTCCACACAGTGAC	140	Feng *et al*. 2013
Pbr003	CAP58021		Trehalose-P-synthase	AGGCGTATGAGACCGTCAAC	AGAACCCGATCTGCATCTTG	147	Feng *et al*. 2013
Pbr018	AAR88383		actin-1	GGGACATCACCGACTACCTG	ACTGCTCCGAGTTGGACATC	150	Feng *et al*. 2013
PbBMST-1			PbBSMT1	Confidential	Confidential	133	Ludwig-Müller, J. unpublis
GH3		evm.model.scaffold542.19 XLOC_009322	GH3-promoter protein	TGGCGTTCAGAATTACGTG	CGTTGGTCAGCACCAGTT	143	Schwelm unpublished
CytOx		evm.model.scaffold478.28 XLOC_008336	Cytokinine Oxidase	GACGTTGCTCACGGTCAT	GAACGGCGTTGATCACAT	119	Schwelm unpublished

PCR program 1

Cycle 1: (1X)
- Step 1: 95.0 °C — 180 sec

Cycle 2: (40X)
- Step 1: 95.0 °C — 10 sec
- Step 2: 58.0 °C — 30 sec

Data collection and real-time analysis enabled.

Cycle 3: (81X) –
- Step 1: 55 °C- 95 °C — 30 sec

Increase set point temperature after cycle 2 by 0.5 °C
Melt curve data collection and analysis enabled.

PCR program 2

Cycle 1: (1X)
- Step 1: 95.0 °C — 180 sec

Cycle 2: (40X)
- Step 1: 95.0 °C — 10 sec
- Step 2: 60.0 °C — 30 sec

Data collection and real-time analysis enabled.

Cycle 3: (81X)
- Step 1: 55.0 °C-95.0 °C — 30 sec

Increase set point temperature after cycle 2 by 0.5 °C
Melt curve data collection and analysis enabled.

PCR program 3

Cycle 1: (1X)
- Step 1: 95.0 °C — 180 sec

Cycle 2: (40X)
- Step 1: 95.0 °C — 10 sec
- Step 2: 58.0 °C — 30 sec
- Step 3: 72.0 °C — 30 sec

Data collection and real-time analysis enabled.

Cycle 3: (81X)
- Step 1: 55.0 °C-95.0 °C — 30 sec

Increase set point temperature after cycle 2 by 0.5 °C
Melt curve data collection and analysis enabled.

Appendix 2

qPCR results

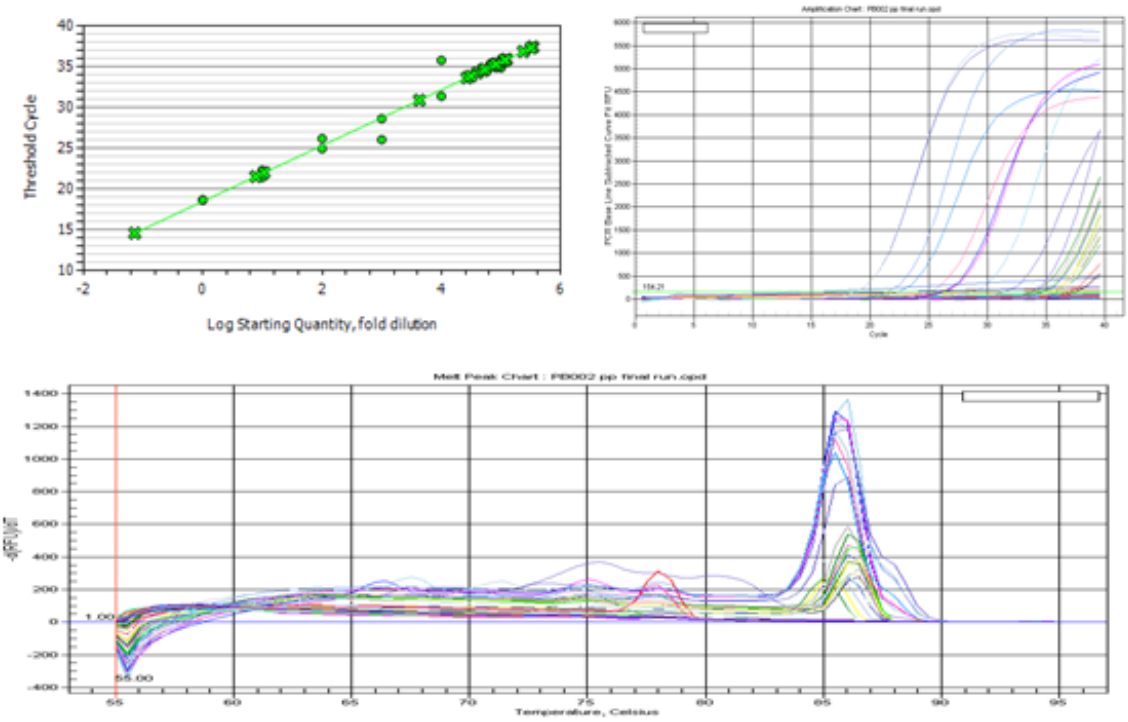

Fig. 1. Standard curve (upper left chart) amplification chart (upper right chart) and melt-peak-chart (lower) of cDNA from the dilution series, cDNA generated from *P. brassicae* inoculated and non-inoculated roots and non-template control amplified with primer pair Pbr002.

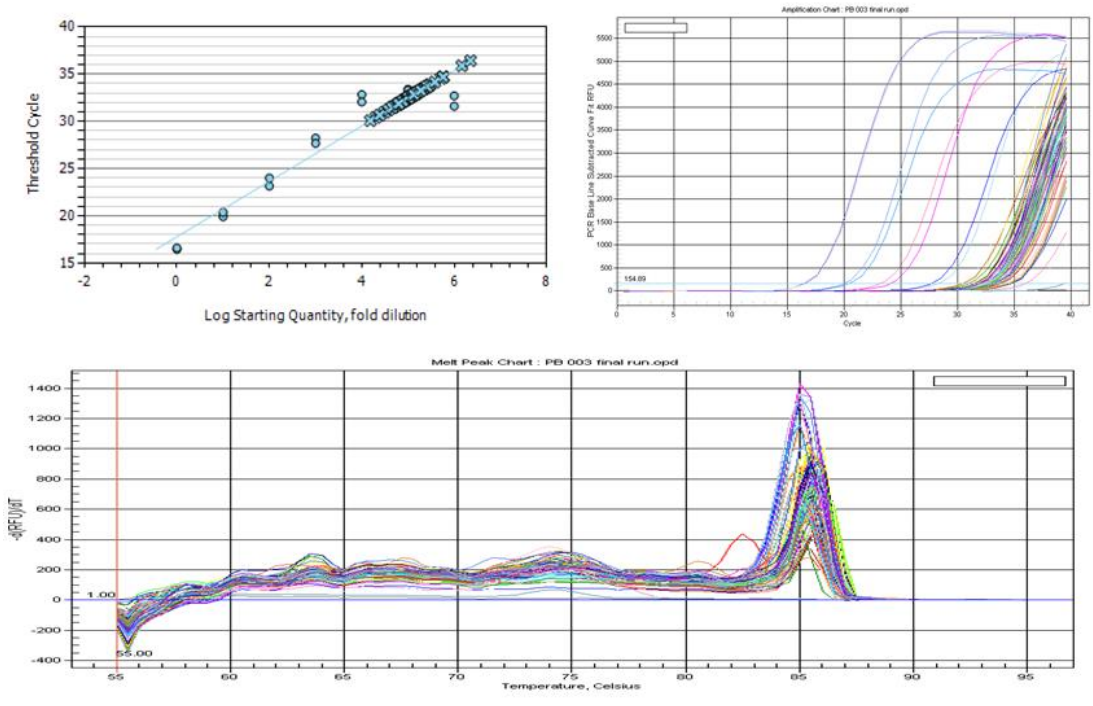

Fig. 2. Standard curve (upper left chart) amplification chart (upper right chart) and melt-peak-chart (lower) of cDNA from the dilution series, cDNA generated from *P. brassicae* inoculated and non-inoculated roots and non-template control amplified with primer pair Pbr003.

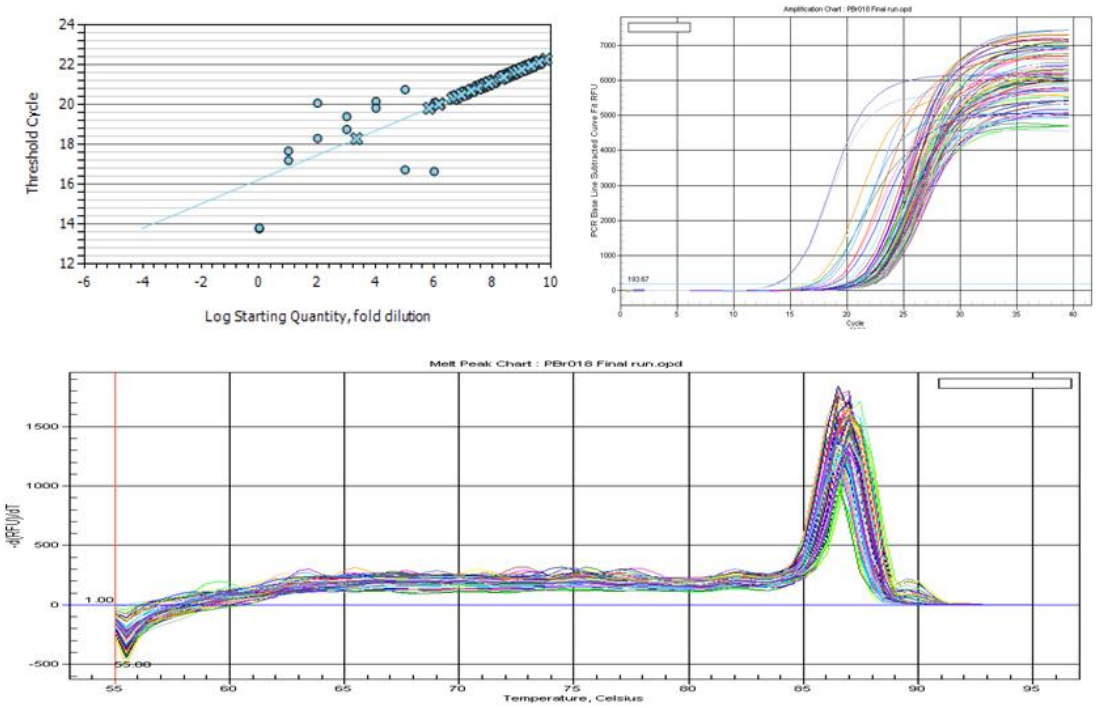

Fig. 3. Standard curve (upper left chart) amplification chart (upper right chart) and melt-peak-chart (lower) of cDNA from the dilution series, cDNA generated from *P. brassicae* inoculated and non-inoculated roots and non-template control amplified with primer pair Pbr018.

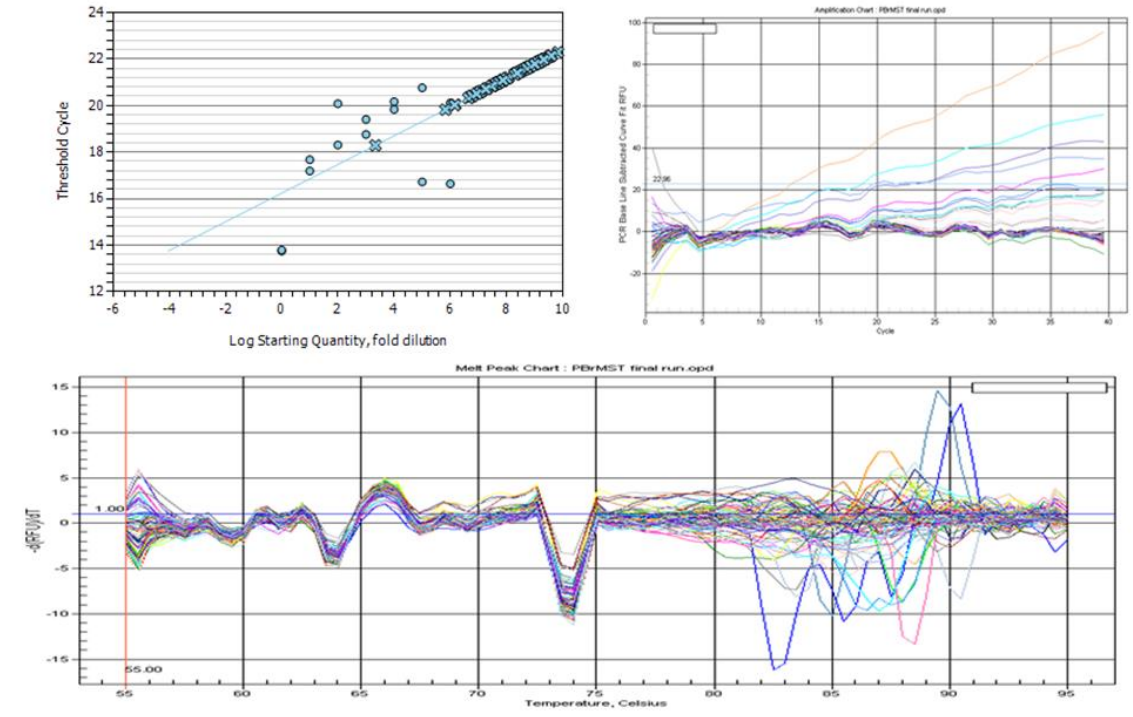

Fig. 4. Standard curve (upper left chart) amplification chart (upper right chart) and melt-peak-chart (lower) of cDNA from the dilution series, cDNA generated from P. brassicae inoculated and non-inoculated roots and non-template control amplified with primer pair PBSMT.

Appendix 3

Phylogenetic analysis of Swedish field isolates

Introduction

Studying phylogeny of *P. brassicae* is of great interest as understanding the distribution and infection routes of *P. brassicae* is important for development and management of disease control measures (Niwa *et al.* 2011). A method for analysis of phylogeny of *P. brassicae* isolates by rDNA polymorphisms had been developed (Niwa *et al.* 2011) and phylogeny of Japanese isolates had been tested.

Aim

The aim of this experiment was to analyse the phylogeny of two Swedish *P. brassicae* isolates together with Japanese isolates.

Materials and methods

Isolate 1

Clubroots from the county of Skåne were surface-sterilised, homogenised and filtered as described in chapter 3.2. Microscopic investigation did not reveal any resting spores after the first gradient centrifugation (chapter 3.2). However, DNA was extracted using Qiagen DNeasy Plant Mini Kit following the manufacturer's protocol.

Isolate 2

Resting spores were extracted from clubbed roots from Hallsberg, county of Örebro. DNA was extracted as described by Russel and Bulman (2005), with the following adaptions. After the lysis step the DNA solution was once extracted with Phenol:chloroform:isoamylalcohol followed by 2 additional chloroform:isoamylalcohol extractions. DNA concentrations were measured using a Nanodrop spectrometer.

DNA samples were analysed by PCR. The PCR programme included 1 cycle at 98°C for 30 seconds, 30 cycles at 98°C for 15 sec, 58°C for 15 seconds and 72°C for 15 seconds followed by 72°C for 5 minutes. Of each PCR solution, 3 µl were loaded onto a gel, which was run at 100 V, 200 mA for 45 minutes.

The DNA samples were analysed by PCR using primers NDL22f (Niwa *et al*, 2011) and 28s4r (Niwa personal communication). The PCR programme included: 1 cycle at 98°C for 30 seconds, 30 cycles at 98°C for 15 sec, 58°C for 15 sec and 72°C for 15 sec followed by 72°C for 5 min. Of each PCR solution, 3 µl were loaded onto a gel, which was run at 100 V, 200 mA for 45 minutes.

Results

The DNA contents of sample 1 and 2 were 22.96 ng/µl and 65.18 ng/µl, respectively (Table 1). The A260/280 ratio was 1.76 for sample 1 and 1.92 for sample 2. The gel electrophoresis revealed that several PCR products of different lengths were obtained for all samples (Fig. 1).

Table 1. DNA concentration and A260/280 ratio of isolate 1 and 2 measured by Nanodrop spectrometer.

Isolate	Sample id	ng/µl	A260/280
1	3	22.96	1.76
1	4	33.48	1.60
2	1	65.18	1.92
2	2	27.95	1.79

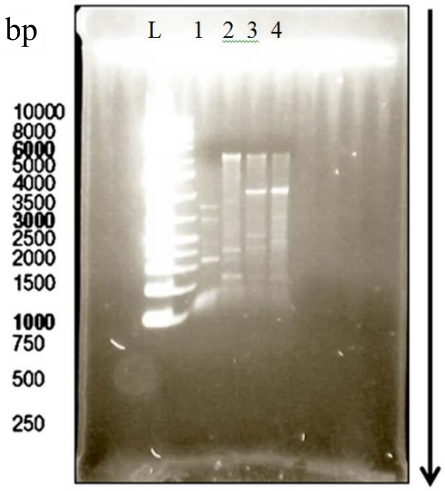

Fig. 1. PCR products from amplification of *P. brassicae* rDNA separated by gel electrophoresis. Lanes 1 and 2 are products of two samples from county of Örebro; Lanes 3 and 4 are products of two samples from county of Skåne; L is DNA ladder. Three visible bands can be seen for sample 1, approximately 1.0, 2.0 and 2.25 kb long, for sample 2 there are three visible bands approximately 0.75, 1.0 and 4.0 kb long, in sample 3 there are 4 visible bands approximately 0.75, 1.0, 1.5, 2.5 and 4.0 kb long and in sample 4 there are four bands of approximately 0.75, 1.5, 2.5 and 4.0 kb.

Discussion

The phylogenetic analysis failed because unspecific PCR products were obtained. These unspecific DNA products could possibly derive from unspecific amplification of DNA or from some contaminations.

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Department of Plant Biology and Forest Genetics
Uppsala BioCenter
Box 7080
75007 Uppsala, Sweden