Persistence of
Plasmodiophora brassicae

Influence of Non-Host Plants, Soil Fauna and Organic Material

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Abstract

Plasmodiophora brassicae, causal agent of clubroot disease of crucifers, has tolerant resting spores that permit its survival in the absence of a host plant. The resting spores are expected to germinate when triggered by specific substances excreted from host plant roots, but they also respond to other cues. As the zoospores emerging at germination are sensitive and short-lived, stimulation of resting spore germination is a potential method for management of clubroot disease.

Certain non-host plants have been found to increase resting spore germination. The effect of four non-host plants on *P. brassicae* was studied in laboratory-, greenhouse- and field studies. In the laboratory, root exudate solution of *Lolium perenne* (Perennial ryegrass) stimulated germination of resting spores more strongly than other non-host plants tested (*Allium porrum*, leek; *Trifolium pratense*, red clover; *Secale cereale*, winter rye) or the host plant *Brassica rapa* var. *pekinensis* (Chinese cabbage). When grown in soil, however, no species-specific effect of any of the plants was observed on the persistence of *P. brassicae*.

It has been claimed that resting spore germination increases in response to substances excreted from decaying plant material or to a general increase in soil biological activity. However, no change in pathogen persistence was observed when infested soil was treated with plant material or with a combination of grass material and earthworms.

A bioassay made it possible to correlate disease severity with concentration of resting spores in soil. This analysis provides a coarse estimation of resting spore concentration, and smaller changes in spore load may not have been detected. Nevertheless, none of the treatments showed an influence that would render them useful in dealing with heavily *P. brassicae*-infested soils within short time periods. The studies emphasise the need for methods for direct and precise quantification of *P. brassicae* in soil. The gaps in our understanding of the life cycle of the pathogen, including the factors that determine its survival, dispersal and germination are important issues that need to be addressed before we can adequately develop management strategies to control *P. brassicae*.

Keywords: *Plasmodiophora brassicae*, clubroot disease, dormancy, root exudate, germination-stimulating factor, resting spore, *Lolium perenne*, earthworm, soil fauna

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Klumprotsjuka på kålväxter - inverkan av sanerande grödor, markdjur och organiskt material på *Plasmodiophora brassicae*

Plasmodiophora brassicae orsakar klumprotsjuka på korsblommiga växter, och är ett stort problem vid odling av kålväxter. Den har tåliga vilsporer som kan överleva mycket länge i marken, något som förklarats med vilsporernas tjocka väggar i kombination med förmåga att gro bara när en värdväxt finns i närheten. De frisimmande zoosporer som frigörs då vilsporer gror är känsliga och kortlivade, och måste snabbt finna en rot att infektera. Om vilsporerna kan stimuleras att gro även i frånvaro av värdväxter ger detta möjlighet att sanera jord från *P. brassicae*.

Förutom värdväxterna anses vissa så kallade sanerande grödor ha groningsstimulerande inverkan på *P. brassicae*. Effekten av sådana växter undersöktes i laboratorie-, växthus- och fältstudier. I vattenlösning stimulerade rotexudatlösning av engelskt rajgräs (*Lolium perenne*) vilsporerna att gro mer än övriga testade icke-värdar: höstråg (*Secale cereale*), purjolök (*Allium porrum*), och rödklöver (*Trifolium pratense*). Den stimulerande effekten var även större än den för värdväxten salladskål (*Brassica rapa* var. *pekinensis*). Vid odling i jord - i växthus- och fältförsök - minskade däremot ingen av växtarterna *P. brassicae* mer än de andra.

Även ämnen som utsöndras från multnande växtmaterial och en ökad biologisk aktivitet har föreslagits minska vilsporernas överlevnad. Men i försök med tillförsel av växtmaterial med eller utan daggmasken *Aporrectodea caliginosa* (lermasken) märktes ingen mätbar minskning av *P. brassicae*.

Med en biotestmetod kunde infektionen av testplantor korreleras med sporkoncentrationen i jorden. Metoden ger ett grovt mått, och det kan inte uteslutas att en eller flera av de olika behandlingarna påverkade överlevnaden i viss omfattning, men att effekterna inte tydliggörs med denna metod. För att detektera mindre skillnader krävs en mer noggrann mätmetod för *P. brassicae* i jord. Vi kan ändå dra slutsatsen att ingen av de undersökta behandlingarna på kort sikt kan förväntas lindra problem med klumprotsjuka i jordar med höga sporkoncentrationer. För att komma framåt i förståelsen av vilsporernas överlevnad behövs både förbättrade metoder för kvantifiering av *P. brassicae* och ökad kunskap om patogenens biologi. Det kan utgöra nyckeln till framtida framgångsrika metoder att bemästra klumprotsjuka.

Contents

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Friberg, H., Lagerlöf, J. & Rämert, B. Germination of *Plasmodiophora brassicae* resting spores stimulated by a non-host plant. *European Journal of Plant Pathology, in press.*

II. Friberg, H., Lagerlöf, J., & Rämert, B. Are non-host plants useful in management of clubroot disease? (Submitted manuscript).

III. Friberg, H., Lagerlöf, J., Hedlund, K. & Rämert, B. Effect of earthworms and incorporation of grass on *Plasmodiophora brassicae*. (Manuscript).

IV. Svensson, K. & Friberg, H. Changes in microbial status as induced by earthworms and grass amendments. (Submitted manuscript).

V. Friberg, H. & Hedlund, K. A bioassay method of detecting *Plasmodiophora brassicae* spore concentrations in soil. (Manuscript).

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Introduction

Plasmodiophora brassicae Woron., causal agent of clubroot disease, is a severe problem in production of Brassica crops. The resting spores have the capacity to survive for at least 15 years waiting for a suitable host (Wallenhammar, 1996). This extended survival has been explained by a consequential dormancy of the resting spores. Spore germination is expected to be induced by specific substances excreted from host plant roots. It has been observed, however, that germination occurs also in the absence of host plant roots, and that it can be stimulated by various environmental factors (Macfarlane, 1970; Suzuki *et al.*, 1992; Takahashi, 1994a, b; Begon, Harper & Townsend, 1996).

The protected life of *P. brassicae* in the soil and inside plant roots makes it difficult to control, and fungicides do not give a complete control of *P. brassicae* (Karling, 1968). Despite extensive breeding programmes for resistant cultivars, the genetic variability of the pathogen has caused problems and few resistant cultivars have been produced. The pathogen has often been able to overcome the resistance after some time (Voorrips, 1995). Clubroot control today is thus restricted to cultural management methods that create an environment less beneficial for disease development, especially avoidance of all host plants, cruciferous crops as well as weeds, in the crop rotation. Although the time for a heavily infested soil to reach a spore concentration below the detection level is unacceptably long from a grower's perspective, a sound crop rotation is the most important way to prevent severe outbreaks of clubroot disease and build-up of *P. brassicae* resting spores. Therefore, increased knowledge about the factors that enhance the rate of spore disappearance is highly interesting. Finding of such factors would facilitate the development of a soil management system that enables Brassica crops to be grown without reaching spore concentrations of *P. brassicae* that damage the crop.

Aims of the thesis

In this thesis, the influence of soil biological interactions on persistence of *P. brassicae* is investigated. Firstly, the influence of non-host plants on germination and persistence of *P. brassicae* was studied (Papers I &II). Secondly, the abundance of soil arthropods around infected roots compared to healthy roots and in soil without roots was investigated. Thirdly, the influence of incorporation of *Lolium perenne* L., alone and in combination with the earthworm *Aporrectodea caliginosa* Savigny, was studied (Papers III & IV). The influence of plant material was further studied in a Master's thesis by Sillén (2003), supervised by Friberg and Rämert, including a comparison of *L. perenne, Brassica rapa* var. *pekinensis* (Lour.) Hanelt and *Trifolium pratense* L.

In all studies on *P. brassicae* in soil, the persistence of the pathogen was measured indirectly, through a bioassay. An investigation was also performed aimed at improving our understanding of how such a method reflects the concentration of *P. brassicae* in soil (Paper V).

Study organism: *Plasmodiophora brassicae*

Plasmodiophora brassicae causes clubroot disease on cruciferous plant species. Among these are several commercially important crops such as broccoli (*Brassica oleracea* L. *Italica* Group), cabbage (*Brassica oleracea* L. *Alba* Group), Swedish turnip (*Brassica napus* L. *Napobrassica* Group) and rape (*Brassica napus* L. *Napus* Group). Some are common weeds like charlock (*Sinapsis arvensis* L.)*,* thale cress (*Arabidopsis thaliana* (L.) Heyhn.)*,* shepherd's purse (*Capsella bursapastoris* (L.) Medik.), and field pennycress (*Thlapsi arvense* L.) (Karling, 1968). Infection by the pathogen results in characteristic swellings on roots and hypocotyls, and aboveground plant parts are often stunted (Kobelt, Siemens & Sacristán, 2000) (Fig. 1). The diseased roots have a limited capacity for uptake of water and nutrients, which often leads to nutrient deficiency symptoms and wilting under dry conditions, and may eventually lead to plant death (Karling, 1968; Siemens *et al.*, 2002).

Fig. 1: *Brassica rapa* var. *pekinensis* with symptoms of clubroot disease (left) compared to a healthy plant (right). Photo: Maria Sillén.

Historically, *P. brassicae* was included in the group of primitive fungi, but it is now grouped into the Protista and the phylum Plasmodiophoromycota (Braselton, 1995). Adaptation to particular host species within a genus or family is common among biotrophic plant pathogens. Based on this, certain species are divided into different *formae specialis*. Such specific adaptation to different crucifer species is not apparent with *P. brassicae* (Crute, 1986). Systems for division into races have been presented (Williams, 1966; Karling, 1968; Buczacki *et al.*, 1975), as well as

descriptions of pathotypes (Fähling, Graf & Siemens, 2003). It is known, however, that pathogenicity of *P. brassicae* is highly variable both between and within field populations, and that differential pathogenicity determinants probably occur with differing frequencies between parasite populations, depending on the plants that have been growing in the soil (Crute, 1986).

In the absence of a host, *P. brassicae* survives as haploid resting spores with a diameter of 3-5 μm. The resting spore is a very resistant structure. Its cell wall (including membrane) consists of approximately 25% chitin, 2.5% other carbohydrates, 34% protein and 18% lipid (Moxham & Buczacki, 1983). Germination of resting spores results in liberation of a biflagellate zoospore (primary zoospore). The frequency of germination increases with spore maturity, and is enhanced by increased humidity and temperature, reduced by alkaline pH, and varies with certain inorganic ions in the soil (Macfarlane, 1970; Takahashi, 1994a). In contrast to the thick-walled resting spores, the zoospores are sensitive to different kind of environmental stress. Zoospores with no access to a host plant are considered to survive for only short periods of time (Karling, 1968; Suzuki *et al.*, 1992; Takahashi, 1994b).

When a zoospore finds its host, it attaches to a root hair and injects its cell contents into the host cell where it develops into a multinucleate plasmodium and later into a multitude of uninucleate zoosporangia from which haploid secondary zoospores are released. The secondary zoospores can again infect root hairs, or infect the root cortex (Ingram & Tommerup, 1972; Mithen & Magrath, 1992). Before infection of cortex, two zoospores may fuse, resulting in a dicaryotic zoospore. It is not known whether this fusion is necessary or whether different mating types of *P. brassicae* exist (Buczacki, 1983; Voorrips, 1996). In the root cortex and stele, *P. brassicae* forms intra-cellular multinucleate plasmodia that stimulate the invaded host cells and adjacent cells to grow and divide through elevated concentrations of cytokinins and auxins (reviewed by Ludwig-Müller, 1999). This leads to the formation of the characteristic galls. During each infection, host DNA sequences are incorporated into the pathogen genome (Bryngelsson *et al.*, 1988). Later, the haploid nuclei in multinucleate plasmodia fuse in pairs. After meiosis, the diploid nuclei develop into haploid resting spores, which after degradation of the galls are released into the soil (Fig. 2) (Ingram & Tommerup, 1972). As many as 10^9 spores per gram of infected root have been recorded (Horiuchi & Hori, 1980; Siemens *et al.*, 2002).

Plasmodiophora brassicae is reported from Brassica producing areas worldwide. The spatial distribution within a field is often aggregated, with the highest spore concentrations found where the diseased plants have grown. Zoospore movement may spread the pathogen in moist soil, but the importance of this is poorly understood since the exact potential of survival time or movement distance is currently not known. It is generally assumed that the zoospores are able to survive only for shorter periods and to migrate only shorter distances, probably rarely more than 5 inches (12.7 cm; Chupp 1917 *cit.* Karling, 1968). Soil organisms can be dispersed by water transport, wind dispersal, soil animals, through root-to-root

contact and through human activities (Dighton *et al.*, 1997; Ristano & Gumpertz, 2000). Karling (1968) argues that dispersal of *P. brassicae* by rain and water is probably more important than wind dispersal. However, the latter may be underestimated with the coarse methods for detection of *P. brassicae* that exist today. It may be that lower concentrations of *P. brassicae* commonly exist in soil, especially in areas of extensive Brassica production, but that these are not noticed unless the soil is repeatedly cropped with susceptible species, and the pathogen is allowed to multiply to concentrations above the detection level (approximately 10^2 spores g^{-1} soil) (Paper IV; Voorrips, 1996; Murakami, Tsushima & Shishido, 2002).
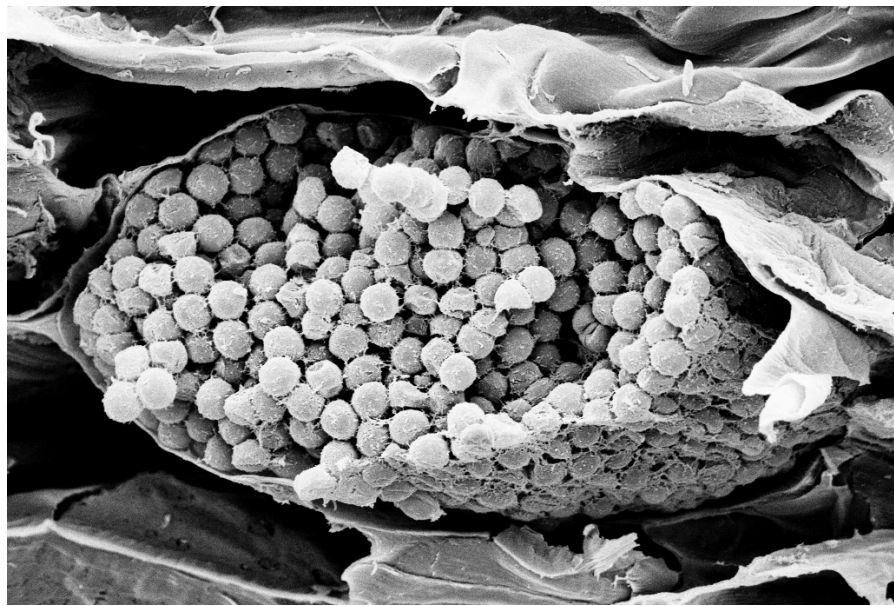

Fig. 2. SEM photo of *Plasmodiophora brassicae* resting spores in infected roots of *Brassica rapa* var. *pekinensis*. Spore size 3-4 μm. Photo: Kerstin Brismar and Salla Marttila.

Despite extensive breeding programmes for resistant cultivars, the genetic variation in the pathogen has caused problems and few resistant cultivars have been produced. In those that have been produced the pathogen has been able to overcome the resistance after some time (Voorrips, 1995). Since no truly resistant cultivars or effective chemical methods are available, cultural practices aimed at reducing pathogen dispersal and propagation are crucial for growers. Besides avoidance of host plant crops and weeds, avoidance of high soil moisture and temperature reduces disease development (Murakami, Tsushima & Shishido, 2002). Liming of the soil to increase soil pH is also widely practised in clubroot control.

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The level of pathogen success and the pathogen's ability to cause disease within a certain soil has led to the concept of soil suppressiveness to the pathogen or to the disease. Characteristics of agricultural soils make them more or less suppressive or conducive to different soil-borne plant pathogens. Some of these characteristics are determined by indigenous factors connected to *e.g.* soil type or climate, while others are influenced by cultural practices (Höper & Alabouvette, 1996; Garbeva, van Veen & van Elsas, 2004). Soil suppressiveness to clubroot has been positively correlated to alkalinity, concentration of Ca, Mg and B (Myers & Campbell, 1985; Dixon & Webster, 1988; Webster & Dixon, 1991; Young, Cheng & Waller, 1991; Höper & Alabouvette, 1996) and phenolic substances such as gentisic acid (Young, Cheng & Waller, 1991). Abiotic factors such as soil texture are of importance (Worku & Gerhardson, 1996) and in some soils also biotic factors (Murakami, Tsushima & Shishido, 2000). With the aim of finding organisms suitable for inundation or inoculation biological control of *P. brassicae*, the influence of specific isolates of microorganisms has been investigated. The fungi *Phoma glomerata* (Corda) Wollenweber et Hochapfel and *Heteroconium chaetospira* (Grove) MB Ellis have both shown promising qualities as biocontrol agents of clubroot disease (Arie *et al.*, 1998, 1999; Narisawa, Ohki & Hashiba, 2000; Narisawa *et al.*, 2005). Both fungi suppress the development of the disease, but the studies were not aimed at finding methods for reduction of resting spore concentrations in infested soils. Information on the effects of ubiquitous soil microorganisms on *P. brassicae* is scarce. Takahashi (1994b) found that viability of *P. brassicae* resting spores was lower in sterilised soils compared to non-sterilised, while Smith (*cit.* Takahashi, 1994b) drew the opposite conclusion. In contrast to the most frequently investigated pathogens in biological control studies, *P. brassicae* is an obligate biotroph, without the ability to survive saprophytically. It is therefore probably less affected by competition from other soil microorganisms. Suppression of clubroot disease could instead be connected to survival or germination of resting spores, interactions with the sensitive zoospores or through influences on the host plants that lead to reduced disease development.

It has been suggested that *P. brassicae* resting spores have the ability to recognise host plant roots and that much of the germination is a specific response to the presence of host plants (Narita & Nishiyama, 1955; Macfarlane, 1970). Cropping of host plants long enough to activate the spores, but removing them before new resting spores are produced has provided some control of clubroot disease (Harling & Kennedy, 1991). This 'bait cropping' is an expensive and time-consuming method of control that is hard to use on a field scale. Besides, this method involves the risk of inadvertently multiplying the pathogen in the event of new resting spores being produced earlier than expected. Similarly to host plants, certain non-host plants have been suggested to increase germination of *P. brassicae* (Bochow, 1965; Ikegami, 1985; Robak, 1994; Rod & Robak, 1994). Commonly, these plants are simply termed 'non-host plants', which is somewhat misleading. 'Host' is defined as 'any organism in which another spends part or all of its life, and from which it derives nourishment or gets protection' (Lawrece, 1995). In some 'non-hosts' for *P. brassicae,* at least the first part of the life cycle,

the root hair phase, can take place. In fact, the observation of sporangia in the root hairs was the reason why they were first suggested to influence *P. brassicae* germination. Also with the definition of a host used by Walker (1969) – 'the plant that gets diseased from presence of a parasitic organism' – the term 'non-host' is misleading since the pathogen can cause disease in some of the plants, at least to some extent. As shown by Ludwig-Müller *et al.* (1999), *P. brassicae* can even use some of these plants for production of viable resting spores, although only to a limited extent. Therefore, the plants are poor hosts rather than non-hosts, and these plants might even be of importance for *P. brassicae* to keep a small population viable in absence of better hosts. In this thesis, I have used the term non-hosts for all non-cruciferous plants.

Effects of different plant species on soil microorganisms

Plant species have an important selective influence on the microbial community structure of the soil. Together with other environmental factors, plants determine which organisms are active and reproduce (Grayston *et al.*, 1998). The effect of plants on soil microbial communities varies with the soil type and the combination of soil type and plant species. Differences in root exudate amounts and composition are likely to affect community structure since microbial species differ in their ability to metabolise and compete for different carbon sources (Marschner Crowley & Yang, 2004). Certain substances may have an inhibitory effect on soil microorganisms, and sometimes on the functioning of the whole microbial community (Innes, Hobbs & Bardgett, 2004; Marschner, Crowley & Yang., 2004). Root exudates may also act as messengers that communicate interactions between roots and soil organisms, sometimes by mimicking different signalling molecules (Walker *et al.*, 2003). Similarly to other soil microorganisms, soil-borne plant pathogens may be selectively influenced by the plant species grown, directly by the different substances found in root exudates, or indirectly through interactions with other soil microorganisms. Such effects of plants on pathogens have been included in the concept of induced soil suppressiveness, which has sometimes been correlated to specific groups of soil microorganisms but is mostly poorly understood because of the complexity of the microbial community (Garbeva *et al.*, 2004).

Soil fauna and plant pathogens

The soil fauna influence soil microbes directly by feeding on them and by dispersing them. They can also alter the physical environment by fragmenting organic material, burrowing through the soil, mixing soil and depositing faeces (Swift, Heal & Anderson, 1979; Brown, 1995; Coleman & Crossley, 1996). For obligate parasites like *P. brassicae*, which lack the ability to survive saprophytically in the soil, animal ingestion of spores and other types of propagules can be of major importance for the population dynamics. Pathogens that survive passage through the gut have the possibility to colonise new habitats if the animal moves in the meantime. This implies that faunal feeding may have a positive effect on the pathogen, even if spore germination is reduced by gut passage, as it may

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expand the distribution of the pathogen and increase the probability of finding a new host (Toyota & Kimura, 1994).

The influence of gut passage on spores and other types of propagules varies from complete survival and even enhanced germination to complete digestion (Moody, Piearce & Dighton, 1996). The factors that determine survival are not fully understood. It might be expected that compared to short-lived propagules, those surviving in the soil for extended periods of time would also be more tolerant to the physical and chemical actions of gut passage. At present, there are too few survival studies on different types of propagules to draw any such conclusion, and the underlying mechanism may also be more complicated than a simple relationship between soil survival and gut passage tolerance (Friberg, Lagerlöf & Rämert, 2005). In addition to propagule features, gut survival probably varies among the different animal groups as they have differing potential to digest substances found in cell walls. One example of this variation is found within the earthworms, where differences in feeding habits are reflected in the composition of digestive enzymes (Urbášek & Pižl, 1991; Doube & Brown, 1998; Brown, Barois & Lavelle, 2000). Another possible important factor in determining the fate of ingested propagules is the gut transit time. This factor is highly variable both within and between animal groups, and also varies with other factors such as temperature and food quality (Hartenstein, Hartenstein & Hartenstein, 1981; Hartenstein & Amico, 1983; Hendriksen, 1991; Daniel & Anderson, 1992; Scheu, 1992; Thimm *et al.*, 1998).

Organic material and plant pathogens

The amendment of cultivated soils with organic material has been suggested to increase the general suppressiveness of the soil, *i.e.* the non-specific type of disease or pathogen suppressiveness resulting from *e.g.* competition (Huber & Watson, 1970; Vandermeer, 1995; Grunwald, Hu & van Bruggen, 2000; van Bruggen & Semenov, 2000; Lazarovits, 2001; Bailey & Lazarovitz, 2003). Amount and quality of soil organic material are factors frequently discussed in relation to the concept of soil health, a term describing the stability of the soil, its resilience, diversity and level of internal cycling of nutrients (Doran & Zeiss, 2000; van Bruggen & Semenov, 2000). Nevertheless, it must be remembered that the factors determining the level of suppressiveness to a disease are complex and depend on the soil type as well as the type and stage of decomposition of the organic material (Huber & Watson, 1970; Grunwald, Hu & van Bruggen, 2000; Lazarovits, 2001; Bailey & Lazarovitz, 2003; Steinberg *et al.*, 2004). Certain ecological adaptations of the pathogen determine which type of changes will suppress or enhance it (Deacon, 1991; Roudriguez & Redman, 1997; Yin *et al.*, 2004). For example, suppression of *Pythium* spp. has been considered to be general in its nature, in contrast to suppression of *Rhizoctonia* spp., which is considered to be of a more specific nature. As a consequence, suppression of *Pythium* damping-off is correlated to saprophytic competition and frequently occurs in decomposition of fresh organic material, whereas suppression of diseases caused by *Rhizoctonia* spp. is due to the establishment of cellulolytic antagonists, and sets in later in the

decomposition process (Grunwald, Hu & van Bruggen, 2000). For obligate parasites like *P. brassicae*, competition from organisms colonising the material is probably not important since *P. brassicae* does not derive nourishment from dead plant material but can only grow inside its host. The effects of different functional groups of microorganisms colonising organic material, and their interaction with *P. brassicae* are poorly understood. It is known, however, that chemical and physical soil characteristics influence both the pathogen and the development of the disease, and that such changes can be the result of applying organic material.

Materials and methods

In this thesis, I worked with two different field isolates of *P. brassicae*, *i.e.* populations of the pathogen isolated from an infested field soil (Papers I, II & III) and from roots of infected plants (Paper V) (Voorrips, 1995). Field isolates like these are expected to have a relatively high level of genetic diversity (Manzanares-Dauleux *et al.*, 2001).

Methods in analysis of *P. brassicae*

The bioassay method (Paper V) was used in the analysis of *P. brassicae* resting spore concentration in soil. The biological background of the bioassay is that disease severity increases with the number of spores that attack the root, and that an early infection of the taproot gives a higher disease score than a late infection of lateral roots. A high spore concentration in the soil increases the probability of an early infection (Voorrips, 1996). Other methods for *P. brassicae* quantification in soil include direct counting of spores under the microscope after staining of the material (Takahashi & Yamaguchi, 1987, 1989). This method is time-consuming and requires specialist equipment and trained personnel. Serological detection and PCR-based methods have been tested, but have not yet been widely used because of problems with genetic variability of *P. brassicae* and lack of quantitative methods, respectively (Wakeham & White, 1996; Faggian *et al.*, 1999; Wallenhammar & Arwidsson, 2001).

  In the laboratory experiment on non-host plants (Paper I), spore reaction to the different treatments was analysed under the microscope after staining with orcein. With this method, the whole non-germinated spores absorb the orcein stain, while the germinated spores only absorb the orcein in the cell wall and appear as empty circles (Naiki, Dixon & Ikegami, 1987). In a study by Sillén (2003) in which spore reaction was analysed in non-transparent solutions of homogenised plant material in water, the root-dip method (Dixon, 1976) was used instead. Bait plant seedlings were placed in the spore solution for 5 minutes, whereafter they were grown according to the bioassay method (Paper V). Similarly to the bioassay method, the results of the root-dip method are influenced not only by the number of spores in the solution but also by the reaction of the test plants to the treatment.

Non-host plants

Four non-host plants were studied for their effect on *P. brassicae.* The study comprised a laboratory experiment, a greenhouse experiment and a field experiment. In the laboratory experiment, germination of *P. brassicae* resting spores was studied in the presence of the different non-host plant species, as compared with a susceptible host and controls without plants (Paper I). In the greenhouse and field experiments, the effect of the same non-host plants grown in soil on *P. brassicae* resting spores was studied, and measured as clubroot disease development in bait plants (Paper II). The hypothesis was that non-host plants could increase germination of *P. brassicae* resting spores, and thereby contribute to sanitation of infested fields. Three of the non-hosts (*L. perenne, Allium porrum* L.*, Secale cereale* L.) have been shown to stimulate germination of *P. brassicae* spores (Ikegami, 1985; Robak, 1994, 1996; Wallenhammar, 1999), while one species (*T. pratense*) is considered not to have this effect (Robak, 1996; Wallenhammar, 1999). In the greenhouse experiment, all plant species were treated equally, while in the field experiment each crop was treated according to its cultural practices. With this design, the effects of the different plant species growing in soil under regulated climatic conditions were studied in the greenhouse during 18 months, while the effects of the same plants growing in the field, in combination with their specific cultural practices, were investigated in a field experiment over three growing seasons. Possible differences in the results obtained from the two experiments were intended to indicate whether the underlying mechanism was an influence of the plant species or an interaction between the plant species and the cropping practices.

In the laboratory experiment (Paper I), the influence of the various chemical variables and plant species on the germination of *P. brassicae* resting spores was statistically analysed by applying partial least squares regression (PLS) to the data set. PLS is a multivariate regression method for modelling relationships between dependent variables and explanatory variables, a regression extension of principal component analysis (PCA). In the PLS analysis, new explanatory variables, the components, are constructed to explain the relationship between the dependent variable and the explanatory variables. Each component is a linear combination of the explanatory variables. Least squares regression is then used to determine equations relating the components to the dependent variable (Eriksson *et al.*, 2001; Anonymous, 2002). In contrast to many other regression methods, PLS is suitable for data sets with fewer observations than variables and a high degree of intercorrelation between independent variables. It is also robust to outliers, and the SIMCA-P software tolerates some missing values of independent variables, as long as the missing data do not have a structure (Garthwaite, 1994; Eriksson *et al.*, 1995, 2001).

Soil fauna

Arthropod abundance around roots with clubroot disease compared with healthy roots and soil without roots was investigated through soil sampling in a clubroot-infested *Brassica napus* L. (Napus Group) field. The hypothesis was that the rich food supply in and around the diseased roots would attract soil arthropods and lead

to increased reproduction, which in turn would result in higher abundances. The field located in Brunnsholm (59°4' N, 17°8' E) outside Enköping 60 km NW Stockholm, Sweden) was sampled in August 2000. Soil samples were collected in the soil matrix, around healthy-looking *B. napus*, and around *B. napus* with symptoms of clubroot disease.

The influence of the earthworm *A. caliginosa* on *P. brassicae* persistence was studied in experimental microcosms in a system with repeated incorporations of organic material. Pathogen reaction was measured as infection of bait plants. The hypothesis was that the earthworms would reduce survival of *P. brassicae* resting spores, leading to a lower disease level in bait plants on earthworm-treated soil. To describe the effect of earthworms on the environment in the microcosms, other components of the soil biota were investigated, with special emphasis on the microbial biomass and metabolic status (Papers III & IV).

Earthworms were collected and recollected by hand sorting (Paper III). Abundance of nematodes, enchytraeids and soil arthropods was estimated in the greenhouse experiment (Paper III) and the field sampling in Brunnsholm, in which soil samples were collected with soil augers. Soil animals were extracted using conventional methods (MacFadyen, 1961; O'Connor, 1962; Sohlenius, 1979). Identification and enumeration were carried out under a binocular microscope with a 40-400 magnification. Collembola were determined to genus or subfamily, Acari were determined to suborder, and Enchytraeidae and Nematoda were enumerated and not further classified.

Organic material

The impact of plant material on *P. brassicae* was studied in microcosm experiments with repeated incorporation of *L. perenne* (Paper III). The study included effects of the material alone and in the presence of earthworms.

Effects of plant material were also studied by Sillén (2003) in a microcosm experiment with a similar design, with incorporation of *L. perenne*, *T. pratense* and *B. rapa* var. *pekinensis*. The direct effect of homogenised plant material of the same species on *P. brassicae* resting spore infectivity was investigated using the root-dip method described above.

Results and discussion

Bioassay

In analysis of *P. brassicae* spore concentrations in soil, the bioassay method (Paper V) was found to be useful but coarse (Fig. 3). It was found to be sensitive mainly in the range of $10^2 - 10^5$ spores g^{-1} soil, and highly variable within the concentration levels. In addition, even a bioassay method that is standardised to minimise the influence of environmental variations and personal judgement in the disease rating cannot alone predict whether a certain treatment decreases disease severity through a reduction in soil inoculum or through other means. The bioassay study emphasises the need for direct methods in the analysis of *P. brassicae* resting spores in soil. In many situations, the combined use of a direct method for spore concentration and a well-developed bioassay would probably be the best choice.

Fig. 3. Brassica rapa var. *pekinensis* bait plants for different spore concentrations of *P. brassicae*. From left: 0, 10^3, 10^4, 10^7, and 10^8 spores g^{-1} soil (d.w.). Disease score 0, 2, 3, 4, and 5. Photo: Jan-Olof Pettersson.

Non-host plants

In the laboratory study (Paper I), root exudate solutions from the non-host plant *L. perenne* stimulated resting spore germination more than exudate solutions from the non-hosts *A. porrum, S. cereale* and *T. pratense*, and more than exudate solutions from the susceptible host plant *B. rapa* var. *pekinensis*. The effect could not be explained by differences in the nutritional composition of the solutions due to differential uptake of the plant species, nor by differences in root activity,

measured as exudation of soluble sugars. Despite this marked influence by *L. perenne* in the laboratory study, there were no differences among the plant species, either in the greenhouse experiment or in the field experiment. In the greenhouse experiment, however, there was a lower infection of bait plants in soil from plant treatments compared to the plant-free control, after soil mixing and decomposition of roots. The effect was seen in all plant treatments and coincided with an increase in pH. Other mechanisms may have been involved as well. If so, they seem not to be connected to specific plant species (Paper II).

It is possible that the positive influence of *L. perenne* on resting spore germination found in the laboratory also occurs in soil, and results in a lower concentration of *P. brassicae* with time, but that this influence was not observed due to the relatively short periods of study (3 years in field and 14 months in greenhouse) in combination with the inability of the bioassay method to detect small changes in spore density. Based on our experiments and literature studies, however, we cannot conclude that any of the plants tested is useful in the sanitation of soils heavily infested with *P. brassicae*.

Soil fauna

In the field sampling in a *P. brassicae*-infested *B. napus* field, more collembolans were found around the diseased roots than in the soil without roots. The healthy-looking roots had an intermediate number of collembolans, not significantly different either from diseased roots or soil without roots (Fig. 4). Most collembolans found were *Folsomia* spp. and species within the subfamily *Tullbergiinae*. The other groups of soil arthropods were not as abundant as the collembolans, and there were no significant differences among the three sampling categories (Fig. 4). Most of the mites found were Tarsonemida. The amount and composition of animals found around a root is determined by the attractiveness of the root and the reproduction of animals in and around that root. Species with a high reproduction rate, *e.g.* Acaridae are probably more determined by reproduction while animals with a slow reproduction rate, *e.g.* Oribatid mites and most collembolan species are probably more influenced by the attractiveness of the habitat.

The sampling was followed by studies on the effect of soil animals on *P. brassicae*. Presence of the earthworm *A. caliginosa* had an effect on the soil environment in the microcosm experiment (Paper III), on the amount of organic material in the soil and on the response of the microbial activity following addition of cut grass to the soil (Paper IV). In contrast to these differences, no decrease in abundance of *P. brassicae* as an effect of *A. caliginosa* could be detected (Paper III).
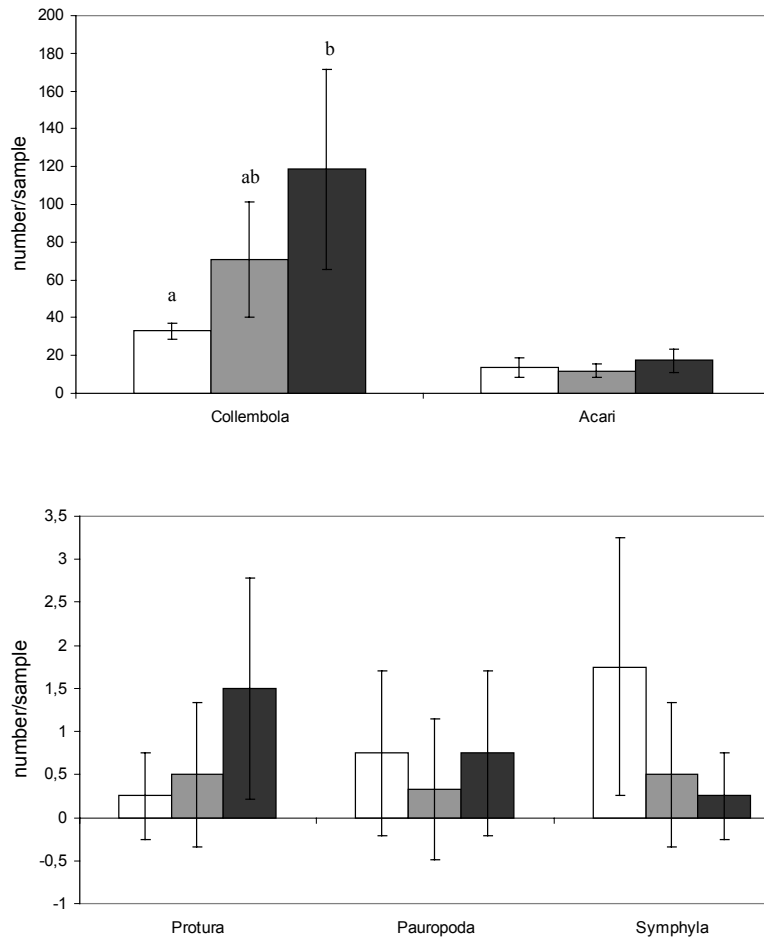
Fig. 4. Numbers of Collembola, Acari, Protura, Pauropoda and Symphyla found in soil (white bars) around healthy-looking *Brassica napus* roots (grey) and *B. napus* roots with clubroot disease (black). Mean ± SD (n=4). Bars with different letters are significantly different (Tukey´s test, P < 0.05).

Organic material

Incorporation of plant material into the microcosms resulted in major changes in biotic and abiotic characteristics of the system (Papers III & IV). Despite this, the incorporation had no effect on reduction of *P. brassicae* with time, measured as clubroot disease severity of the bait plants. Disease severity as a mean over all nine sampling occasions was higher with grass incorporation compared with the control treatment without grass incorporation. This may indicate either that the grass material stimulated disease development, or that spore germination increased. In the latter case, the increased spore germination with time would result in reduced

survival of resting spores. Such a reduction was not observed during the course of the microcosm experiment.

In similar systems studied by Sillén (2003), no effect of plant material incorporation could be detected either at sampling directly after incorporation or as a long-term effect (12 weeks) from a total of 3 additions of 250 g fresh plant material per box (approx. 0.3% d.w.) on each occasion. In the root-dip test, the bait plants (*B. rapa* var. *pekinensis*) for the treatment with homogenised material of *B. rapa* var. *pekinensis* did not develop any symptoms of clubroot disease, while the bait plants for the other treatment developed severe symptoms (Sillén, 2003). From this study, it is not possible to determine what the underlying mechanism was. Brassica species are known to contain substances that are toxic or harmful to a wide range of organisms (Kirkegaard & Sarwar, 1998; Sarwar *et al.*, 1998). The treatment may have influenced the resting spores or zoospore survival and ability to infect the roots. It is also possible that the treatment caused damage to the plant roots, resulting in death of root cells and thereby depriving the pathogen of infectable tissue. The observation is interesting and encourages further studies on the effect of Brassica material on infection by *P. brassicae*.

Concluding remarks

This series of studies demonstrates the ability of *P. brassicae* to persist in soil under varying conditions. Despite the effect by *L. perenne* on germination of resting spores observed in water solution (Paper 1) and induced infection of bait plants through treating the spores with plant material of *B. rapa* var. *pekinensis*, no effect on persistence of *P. brassicae* was observed (Papers II & III). As discussed, it is possible that the bioassay method used (Paper V) is too coarse for detection of *P. brassicae* in soil if the treatments only induced minor changes in spore survival.

As it stands today, there are major gaps in our knowledge about the biology of *P. brassicae*, including factors that determine pathogen germination, survival and dispersal, all crucial things for understanding the population dynamics. Research about *P. brassicae* would benefit from access to direct methods for quantification, which would give the possibility to get an accurate measurement of spore concentrations in soil, and make it possible to separate effects on the pathogen from effects on the disease development. In addition, long-term experiments are of particular interest to answer general questions about the persistence of long-lived resting spores, which form part of the life cycle of many soil organisms.

Studying the behaviour of an organism in the laboratory with the aim of predicting its behaviour in a natural environment always involves the risk of erroneous results due to an atypical chemical or biological situation. In the laboratory study on non-hosts (Paper 1), we tried to avoid some of these problems by growing the plants with a controlled nutrient supply, and with the PLS analysis separating out the influence of plant treatments and some possibly important parts

of the chemical composition of the solutions. If the stimulation of germination is a substance-specific reaction, I believe that the results obtained represent processes that are of significance also in the soil, but that the response is enhanced by the favourable conditions for germination (pH, temperature, humidity). Based on this, I regard *L. perenne* of special interest for future studies on *P. brassicae* germination and persistence.

Neither the non-host plants investigated, presence of *A. caliginosa* or incorporation of the plant material in these studies showed an influence that suggests their usefulness in dealing with heavily *P. brassicae*-infested soils within short time periods. Thus, for growers of Brassica crops, the results in this thesis further emphasise the importance of a good crop rotation, even before any clubroot disease is detected, to prevent build-up of spores in the case of undetected disease, and the need for regular inspection of Brassica crops to detect the presence of *P. brassicae* as early as possible.

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'… *whether or not a realistic and lasting control measure is forthcoming in the next few years, there can be no doubt that this most challenging and intractable of organisms will continue to fascinate and frustrate pathologists and mycologists for a great many more years to come*' (Buczacki, 1983).