Studies of the Persistence of Red Clover Cultivars in Sweden

with Particular Reference to *Sclerotinia trifoliorum*

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Abstract

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Clover rot (caused by *Sclerotinia trifoliorum*) and its influence on the persistence of red clover (*Trifolium pratense*) was studied in this thesis. Twenty-one red clover cultivars, of various types, were examined in both field and controlled environment experiments. Field experiments were performed at six sites in different climatic regions. The prevalence of root rot, caused by a broad soil-inhabiting group of secondary invading fungi, including *Fusarium* spp, was also studied.

Both diseases were found to be prevalent throughout the country and disease severity was unaffected by latitude. The late flowering clover cultivars grown in southern Sweden exhibited lower levels of mortality due to clover rot than the earlier heading cultivars. In addition, lower root rot disease indices were recorded for the late flowering cultivars. Tetraploid cultivars grown in northern Sweden exhibited lower levels of mortality due to clover rot than diploid ones. This pattern was not found in southern Sweden or in the controlled environment experiments, where conditions were optimised for clover rot infection. In contrast, tetraploid ones had greater root rot disease indices than diploid cultivars in most cases.

Studies of more than 250 *Sclerotinia trifoliorum* isolates revealed a high level of genetic variation. In laboratory tests, twenty of the isolates exhibited different capacities to cause disease. Fungal strains from northern Sweden generally caused more plant death, but aggressive strains were also found in southern Sweden.

The fungus *Coniothyrium minitans* was shown to control clover rot infection in cultivar SW Torun at a northern site with heavy natural soil infestation.

Key words: *Trifolium pratense*, clover rot, *Fusarium* root rot, disease incidence and severity, cultivar resistance, *Coniothyrium minitans*.

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Front cover: A red clover plant with the leaflets covered by small local infections caused by the ascospores released from the brownish apothecia of *Sclerotinia trifoliorum*.
All photographs within this thesis were taken by the author, unless otherwise stated.

Contents

Appendix

This thesis is based on the following papers, which are referred to by their corresponding Roman numerals:

I. Öhberg, H., Ruth, P. & Bång, U. 2008. Differential responses of red clover cultivars to *Sclerotinia trifoliorum* under diverse natural climatic conditions. (Plant Pathology, Doi: 10.1111/j.1365-3059.2007.01822.x).

II. Öhberg, H., Ruth, P. & Bång, U. 2005. Effect of ploidy and flowering type of red clover cultivars and of isolate origin on severity of clover rot, *Sclerotinia trifoliorum*. Journal of Phytopathology 153, 505-511.

III. Öhberg, H. & Bång, U. 2008. Biological control of clover rot on red clover by *Coniothyrium minitans* under natural and controlled climatic conditions. *Submitted*.

IV. Öhberg, H. & Bång, U. 2008. Occurrence of *Fusarium* root rot complex in various red clover cultivars under different natural climatic conditions. (Manuscript).

Introduction

Red clover is a native of Sweden but it has also been cultivated for at least 200 years. Today in Sweden, red clover is the predominant cultivated forage legume. It is grown throughout the country, mainly in association with grasses, for the production of forage for cattle. In 2006, 1 055 063 ha of land in Sweden was under ley (short-term grassland) and pasture, amounting to approximately 40 % of the total arable land (Statistics Sweden, *Statistical Yearbook of Sweden 2008*). Forage from leys is the most common crop in the northern part of the country, where it covers around 57% of the agricultural land. There are several benefits of adding red clover to the ley compared with a pure grass crop: i) higher yields can be achieved using the same amount of nitrogen (N) fertilizer (reduced need for N-fertilization); ii) the vegetation contains more protein and better relative proportions of K, Mg and Ca; iii) a tastier forage is produced, leading to higher voluntary consumption by cattle and thus iv) higher milk-yield or live weight gains are achieved; v) there is higher soil fertility and N-availability for any subsequent crop and vi) the soil structure is better (Frame, Charlton & Laidlaw, 1998; Ericson, 2005). In organic farming, nitrogen-fixing species are key crops and the government aims to increase the area used for such production. Thus, the persistence of red clover is of great interest. Plant losses due to diseases during the winter are often confused with physical damage and the extent and economic importance of these diseases are, therefore, poorly recorded. In a recent survey, the persistence of a number of red clover cultivars (cvs) at various sites was recorded, but the cause of plant death was not defined (Pulli *et al*., 1996).

This thesis describes studies of diseases that impair the persistence of red clover in leys. The main emphasis is on clover rot caused by *Sclerotinia trifoliorum* (I, II, III), but root rot caused by a broad soil-inhabiting group of secondary invading fungi, of which *Fusarium* spp. are considered the most important, is also included (IV). Both diseases cause plant death, mainly in late winter and spring, during the winter dormancy of the host. Questions regarding the influence of varietal characters, such as ploidy and flowering type, and the impact of hardening conditions on disease development are considered, as well as the occurrence of natural soil infestation at different sites in Sweden. In addition, the possibility of controlling clover rot under typical cultivation conditions by using the fungal antagonist, *Coniothyrium minitans*, was studied at a site with a heavy natural *S. trifoliorum* soil infestation.

In addition to the broad issues mentioned above, several more specific questions arose that needed to be addressed. Previous investigations had found that red clover cultivars differed in their resistance to clover rot based on the location at which they occurred naturally. This might indicate the existence of local strains of *S. trifoliorum*, differing in their ability to cause disease. An investigation into the mycelial compatibility of 253 isolates was performed to provide an indication of their genetic diversity (Study A).

When selecting for resistance to clover rot, breeders in Sweden use coarsely ground grain inocula containing sclerotia to inoculate hardened clover plants. The same procedure was used in the studies described in Paper II. Under natural conditions, however, the plants are infected by both ascospores and mycelium, see below. In a small study, the red clover cultivar (cv.) Björn was subjected to various hardening regimes and inoculated using either ascospores or mycelium, in order to study the influence of these procedures on plant survival (Study B).

There is limited data on the susceptibility to clover rot of other legumes with the potential to be cultivated in Sweden. A small investigation was therefore conducted, including nine species of forage legumes (Study C).

In addition to the studies already mentioned, a description of the host, the pathogens, and a summary of major Swedish work examining *S. trifoliorum* and the root rot complex is presented. Previously, most of these data have only been reported in Swedish.

Finally, there is a discussion and an assessment of potential future research within this field.

The studied host plant; red clover

Red clover is a relatively short-lived perennial, originating from southeastern Eurasia; it is widely distributed across the temperate zones of the world (Taylor & Quesenberry, 1996). Red clover is considered to be native to Sweden and was scientifically named by Carl Linnaeus in 1753, but was first described in Sweden in 1658 by Rudbeck in the *Catalogus plantarum* (Nordstedt, 1920). Cultivation involving ploughing meadows and re-sowing with forage species in planned crop rotations began in the late 18th century in the southern part of Sweden; the same system came into use in the north a century later (Hagsand & Wik, 1968).

Red clover lives in symbiosis with *Rhizobium leguminosarum*, a nitrogen-fixing, nodule-forming bacterium. In a *T. pratense* monoculture in Sweden, the amount of fixed nitrogen (N) varies between 2.2 and 8.5 g N m^{-2} $year^{-1}$ over four years (Carlsson *et al*., 2005).

Red clover is a diploid species with gametophytic self-incompatibility (Taylor & Quesenberry, 1996), *i.e.* it needs to be cross-fertilised before it can set seed. The plant grows from a crown consisting of an accumulation of basal buds at, or slightly above the soil surface. From the crown, the stems elongate and become erect, usually in a branched form, reaching a height of 20–80 cm. The leaves are alternate and trifoliate (with three ovate leaflets), green and often with a characteristic pale "V" in centre. The inflorescence is most commonly pink or purple, and forms a capitilum or head with up to 300 florets, at the ends of the main and axillary stems (Figure 1). The florets are pollinated by bumblebees and

honeybees. Red clover plants usually have a well-developed taproot, which can extend to a depth of more than 1 m. The taproot is somewhat branched; adventitious or lateral roots emanating from the crown also exist (Taylor & Quesenberry, 1996; Frame, Charlton & Laidlaw, 1998).

Figure 1. *Trifolium pratense*: stem, leaves and flowers (Reprinted from Lindman (1917). This figure was originally published in 1917, and therefore copyright has now lapsed.)

Seedlings are susceptible to frost damage, but cold hardiness often increases with plant age. Seedlings of genotypes that set their flowers before the autumn are less winter-hardy than genotypes that remain in a vegetative state throughout the year of seeding (Smith, 1957; Christie & Choo, 1991), indicating that winter hardening is associated with the plants' physiological state. However, flowering itself is not a problem if there is sufficient time afterwards for hardening of the plant to occur. Cold hardening is induced by low temperatures (slightly above 0°C), sometimes in combination with shortened day-length in the autumn (Umaerus, 1963; Frame, Charlton & Laidlaw, 1998). Plants are dormant throughout the winter.

Red clover growth responds to day length. At least 14-hour days are required for the transition from the vegetative to the generative stage in early flowering types

(Smith, 1957) and later heading cultivars require even longer photoperiods (Andersson, 1986). This difference has allowed red clover varieties/cultivars to be split into early-, medium–late- or late-flowering types. Early types of red clover are adapted to shorter day lengths compared with later flowering ones. If a short day cultivar is grown under longer day conditions, flowering will commence too early and if flowers are produced repeatedly during the season, winter hardiness will be compromised (Andersson, 1986). In Sweden, cultivars produce their highest yields in a specific geographical area. If a cultivar is grown to the south or north of this area, the growth habit changes and the cultivation value of the cultivar decreases (Julén, 1997). The early-flowering types were previously referred to as 'double-cut' or medium red clovers in the USA and 'cowgrass' in New Zealand (Frame, Charlton & Laidlaw, 1998). Early red clovers develop early in the spring and exhibit rapid regrowth after cutting, thus producing two growth flushes during a single growing season (Andersson, 1986; Frame, Charlton & Laidlaw, 1998). Medium–late types begin their development later in the spring; they flower two to three weeks later and their regrowth is slower, but they have much better winter hardiness than the early cultivars (Andersson, 1986). The late types begin their spring growth even later and exhibit weaker regrowth, but they are sufficiently winter hardy to be cultivated in the central and northern parts of Sweden (Andersson, 1986). The late-flowering types, previously referred to as 'single-cut' in the USA (Frame, Charlton & Laidlaw, 1998), thus have only one main growth flush.

Originally, red clover was diploid, but since 1939 when the first tetraploid red clover plants were produced, cultivars have been subjected to autopolyploidy in order to increase herbage yields (Sjödin & Ellerström, 1986). Nationally bred tetraploid cultivars have been available on the Swedish market since 1957 (Julén, 1997). Today, tetraploid and diploid cultivars of both late and medium–late flowering types are cultivated in Sweden.

Red clover is predominantly grown in association with companion grasses, except when the aim is seed production (Frame, Charlton & Laidlaw, 1998). In southern Sweden, leys are often harvested for two or three years after sowing and two or three times each summer. In central Sweden, two cuts a year result in higher yields than three cuts, but with lower crude protein and energy contents (Wallgren & Halling, 1995). In northern Sweden, leys tend to be harvested for three or four years after sowing, commonly with two cuts each year (Ericson, 2005).

When establishing the crop, red clover can be sown either with, or without a nurse crop. Common nurse crops in Sweden are spring barley or an oat–pea mixture; these are cut at the end of their growing season, some time before the red clover plants start the process of winter hardening. These crops often help to establish the forage crop by competing with weeds during the first year. The stubble may also help to prevent frost damage during the first winter and the nurse crop may be of economic benefit, since it provides a crop to harvest in the year of clover establishment.

The pathogen *Sclerotinia trifoliorum* and the disease clover rot

The first reported case of clover rot was from Britain in 1849 (Lawes & Gilbert, 1860, according to Scott, 1984); in the mid 1870s, the disease was reported from Germany and Denmark (Eriksson, 1880). The first report from Sweden was in 1878, after major losses of red clover plants at some locations in southern Sweden (Eriksson, 1880). The casual agent was identified as a fungus already recorded in Germany under the name *Peziza ciborioides*. Eriksson (1880) analysed previous published records relating to the disease, transferred the casual fungus into to the taxonomic genus *Sclerotinia*; he renamed it *S. trifoliorum*, which it has been called since then.

Clover rot has long been considered one of the most destructive diseases of clover in northern Europe, however the disease is found in temperate areas all over the world, especially in regions with mild winters or heavy snow covers (Hanson & Kreitlow, 1953). It can occur in all overwintering forage legumes, but currently red clover is the most susceptible host of significant economic importance. In Sweden, the disease is found across all parts of the country where red clover is grown: from a latitude of 55° to 67°N (Ekstrand, 1945). It was one of the major causes of the failure of red clover stands in earlier surveys (Björling, 1939; Lundin & Jönsson, 1974).

Sclerotinia trifoliorum is an ascomycete, an inoperculate discomycete which produces sclerotia *i.e.* the fungal resting structures of *Sclerotinia* spp. Mature sclerotia consist of a black carbonaceous rind and a white medulla of varying size (0.3-10 mm), from which brownish fruiting bodies, apothecia, can develop (Figure 2).

Figure 2. Fruiting bodies, apothecia, of *Sclerotinia trifoliorum* developed from the black resting bodies, sclerotia.

Sclerotinia trifoliorum differs from other *Sclerotinia* spp in that it produces its apothecia in late summer and autumn, in contrast to *S. sclerotiorum*, which produces apothecia in late spring and summer (Williams & Western, 1965a; Kohn, 1979). In addition, *S. trifoliorum* exhibits bipolar heterothallism. There is 4:4 segregation between small and large ascospores within each ascus (Figure 3), a character unique to this species within the genus *Sclerotinia* (Kohn, 1979).

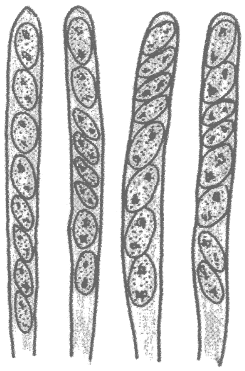

Figure 3. Asci of *Sclerotinia trifoliorum*, each containing eight ascospores. The distribution of the four large and four small spores varies. (After Uhm & Fujii, 1983a in Öhberg, 2004).

Both spore types have the ability to cause infection, exhibit mycelial growth and form sclerotia, but only isolates derived from the large spores are homothallic and can produce apothecia without mating (Uhm & Fujii, 1983a). *S. trifoliorum* mycelia from both spore types produce microconidia that are unable to infect plant tissue (Björling, 1942) but which function as spermatia (Uhm & Fujii, 1983b). If mycelium from a small spore mates with spermatia from a large spore mycelium, apothecia are produced and the isolates generated from the progeny generally differ genetically from both their parental isolates and from each other (Rhenstrom & Free, 1993). In addition, genetically different *S. trifoliorum* isolates are known to differ in their pathogenecity. If a legume cultivar is inoculated with different isolates, differences in resistance are found depending on which isolates are used (Björling, 1939; Dixon & Doodson, 1974; Halimi, Rowe & Pratt, 1998).

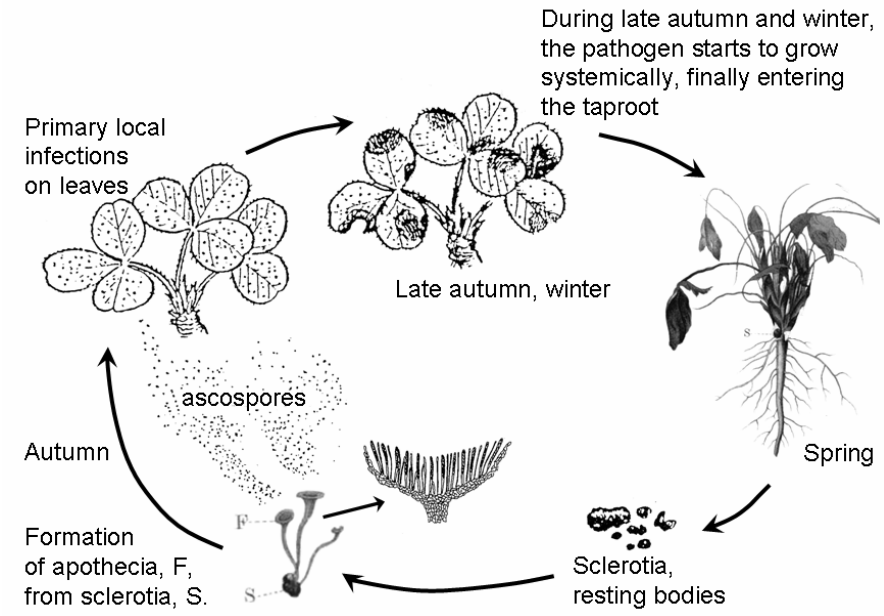

Figure 4. The development of clover rot infection in red clover plants (Modified from Kirchner & Boltshauser, 1897; Raynal, 1985).

Many authors (*e.g.* Eriksson, 1880; Björling, 1942; Dillon Weston, Loveless & Taylor, 1946; Loveless, 1951; Raynal, 1985) have described the life cycle of *S. trifoliorum* and its infection of plants (Figure 4). In brief, apothecia of *S. trifoliorum* develop from soil borne sclerotia in late summer and autumn. The released ascospores, spread by the wind, infect leguminous plants and cause small black local necrotic spots on stems and leaves (Figures 4, 5). The infection remains in a local state until weather conditions favourable to the fungus occur, *i.e.* high humidity and low temperatures above 0 °C, preferably after a frost or snowfall, between late autumn and early spring. The fungus then starts growing systemically within the host (Dijkstra, 1966). The leaves turn olive-greyish-brown before they wither and become covered with mycelium. The mycelia grow through the petioles and stems into the crown and eventually down into the taproot (Hesselman, 1962).

Clover rot infection may also spread between plants by means of mycelial growth, thus creating patches of dead plants in the field. In spring, infected and dead plants are covered by greyish mycelia, with black sclerotia forming in dead plant tissue at soil level, or in the soil close to dead plants (Figures 4, 6).

Figure 5. The brownish apothecia of *Sclerotinia trifoliorum* found in the autumn and red clover leaflets covered by small local infections.

The greatest mortality in red clover stands due to clover rot occurs in late winter and spring and disease symptoms are often mistaken for physical damage by farmers (Dillon Weston, Loveless & Taylor, 1946; Hanson & Kreitlow, 1953). Clover rot infection often results in patches of dead red clover plants that are not obviously linked to topographical differences within the field, as would be the case if killed by ice or water. Since incidence and severity of clover rot is dependent on weather conditions during the autumn and winter, the magnitude of plant losses varies greatly from year to year (Dillon Weston, Loveless & Taylor, 1946; Loveless, 1951; Dijkstra, 1964)

Figure 6. Dead red clover plants covered with a typical greyish *Sclerotinia trifoliorum* mycelium in springtime, some clover plants have escaped the attack and remain viable.

Sclerotia develop during the systemic phase of infection and can be observed in spring after snow melt. In dead plants, sclerotia often develop within the taproots, a few centimetres below the soil surface. The crown and upper parts of the taproot are then disconnected from the lower parts of the taproot (Figure 7). When such a plant is lifted up, sclerotia are readily found in the resulting hole.

Sclerotia are well adapted for survival and can persist in a viable state for long periods. Under experimental conditions, Pape (1937) found viable sclerotia after 7½ years' burial; Dillon Weston, Loveless & Taylor (1946) mention several serious outbreaks of clover rot on land where no susceptible crops had been grown for at least ten years. Soil conditions may influence the viability of sclerotia and the poorest rates of survival have been recorded in dry or flooded soils (Dillon Weston, Loveless & Taylor, 1946). Sclerotia on the surface of the soil produce more apothecia than those at greater depths. Sclerotia found at depths greater than 6–7 cm, only rarely produce apothecia (Björling, 1942; Williams & Western, 1965b).

Previous Swedish studies of *Sclerotinia trifoliorum*

Sclerotinia trifoliorum has been the subject of a number of major studies in Sweden, published only in Swedish. Therefore, I take this opportunity to present a brief summary of these works.

As mentioned above, Eriksson named *S. trifoliorum* in 1880. In his work, he described the appearance of the symptoms on plants, the formation of sclerotia in soil and plant tissue, as well as the formation and structure of apothecia and ascospores.

In 1939, Björling presented his first work on clover rot; he wanted to ascertain whether differences recorded in the field between red clover cultivars with respect to their ability to resist clover rot could be reproduced experimentally. He used three isolates of *S. trifoliorum* from various geographical origins for mycelial inoculation of twelve red clover varieties and obtained a good correlation between

the susceptibility observed in his field and laboratory experiments. He also found differences between the isolates with respect to their ability to cause disease; he thought that this indicated the presence of 'physiological groups' of *S. trifoliorum* with different pathogenecities.

In 1942, Björling published another major work on *S. trifoliorum*, attempting to fully describe the vegetative and sexual development of the fungus and, if possible, to clarify its genetic variation and the possible occurrence of different biotypes. In his work, he found that apothecia were able to develop from sclerotia found at a maximum depth of 7 cm in the soil and that the formation of apothecia was dependent on specific light levels. To elucidate the morphological and histological development of apothecia further, an extensive study of the formation of ascospores and their characteristic spore size segregation, with four large and four small ascospores formed within each ascus, was included. Björling (1942) also discussed the occurrence of 'sterile' sclerotia, *i.e.* the ones unable to produce apothecia, but he did not link the 'sterile' isolates with the small spores, as later discovered by Uhm & Fujii (1983a).

Björling (1942) also refused to acknowledge that microconidia acted as functional spermatia, even though he performed successful mating between 'sterile' and 'fertile' isolates. In conclusion, he found that *S. trifoliorum* appeared readily as new unique isolates and that isolates could be grouped into certain distinct 'pathogenecity groups'. Some locally grown populations of red clover were considered very resistant to clover rot but when grown elsewhere they were found to be susceptible. He suggested that the local red clover was highly resistant because of selection resulting from exposure to all the strains of *S. trifoliorum* present within the local area and that the expression of resistance failed when a previously unknown pathogenecity group of the fungus was encountered. The theory of local adaptation of red clover cultivars to isolates of different geographical origins was, subsequently, widely discussed. Because such a phenomenon would influence selection for resistance, this question was addressed in Papers I and II, using large numbers of both cultivars and isolates.

In 1962, Hesselman presented a licentiate thesis in which he examined the relationships between the age, size and morphology of the red clover plant and *S. trifoliorum* mycelial growth rate and development. He worked mainly with inoculated cloned plant material. Hesselman (1962), found that in resistant clones, the *S. trifoliorum* infection stopped within the crown tissue prior to reaching the central root tissues, and that the infection spread much more slowly than in susceptible ones. He also concluded that selection for resistance in young unhardened plants did not enhance the resistance of offspring compared to the offspring of unselected plants of the same origin. If older, hardened plants were used, however, increased resistance in the progeny was achieved. Plant size was weakly positively correlated to resistance. Hesselman found no link between either plant age or plant morphology and resistance and concluded that red clover resistance to *S. trifoliorum* was rather unspecific and was controlled by factors other than those that he considered.

The root rot complex

Common root rot in red clover is caused by species of *Fusarium* and several other soil fungi and can be considered to be a group of diseases that all produce similar symptoms in plants and often attack simultaneously (Ylimäki, 1967), (Figure 7). The name of the disease complex varies, but *Fusarium* root rot is the common name besides root rot. These fungi are often weak pathogens and cause damage to plants that are already weak or injured. Common root rot kills plants in all stages of development, but the proportion of plants killed by root rot increases with plant age. Plant losses in springtime occur when weakened plants have low food reserves or winter injuries. The disease is found wherever clover is cultivated, but adapted and resistant cultivars and vigorous plants are less susceptible.

Figure 7. Four dead red clover plants from a field experiment. The two plants to the left show symptoms of clover rot: the lower part of the taproot is often separated from the rest of the plant. The plants to the right show root rot symptoms: intact taproots are frequently found and often have a spongy structure.

The pathogens of the root rot complex are abundant in soil and consist of *Fusarium* spp, with *F. avenaceum*, *F. culmorum* and *F. graminearum* being the dominant species, along with *Cylindrocarpon destructans* and *Phoma* sp. Also many other species are involved (Ylimäki, 1967). These fungi vary in their pathogenicity and can occur in leys from the time that they are established. They can cause damping-off of young seedlings. In a cold climate, root rot is considered to be a winter disease and to cause the death of weak plants during their winter dormancy. Nevertheless, the pathogens can also cause considerable plant death during damp and cold conditions occurring in the growing season (Rufelt, 1979). Susceptibility often increases with plant age, and attacks are often more severe in wheel tracks. Since most of the root rot fungi are weak pathogens they often need plant tissue to be damaged to allow them access and cause infection in the red clover plant. The disease most frequently occurs in the primary and secondary roots located 5–7 cm below the crown. In the early stages of infection, the symptoms may be limited to lesions and discolouration of only the cortical region,

but as infection progresses the internal vascular cylinder is damaged and finally destroyed (Ylikäki, 1967), see Figure 8.

Swedish studies of root rot in the past

Rufelt presented his thesis on *Fusarium* root rot of red clover in 1986; this was the first investigation undertaken in Sweden. His research began with a survey of the prevalence of root rot on red clover, demonstrating that it was very common in Sweden and occurred throughout the country (Rufelt, 1979). The disease development was faster in southern than in northern Sweden. He stated that this was due to differences in climate, cropping intensity and cultivars used. He concluded that factors that weaken plants and decreased carbohydrate reserves, such as cutting the ley more frequently and cutting late in the autumn, increased the likelihood of the disease occurring. He assumed that late cultivars, with a slower rate of regrowth and higher carbohydrate levels in the roots, would be less vulnerable to attack by the low-carbohydrate *Fusarium* fungi. He developed a scoring method for disease attack, including an index of external and internal symptoms. The scores range from healthy roots, 0, to severely attacked, 4 (Figure 8). The same fungi were isolated in association with both types of symptoms. In experiments with chemical controls, he concluded that spraying with fungicides was not an effective way of reducing the disease (Rufelt, 1980).

Figure 8. Internal symptoms of split red clover roots more or less damaged by root rot. Scores according to Rufelt (1979). Photo: SW Seed, used with kind permission.

Finding and breeding for persistence in red clover, *Trifolium pratense*, in Sweden

In the late 19th century, the first investigation into resistance to *S. trifoliorum* was conducted in Sweden. The persistence of red clover produced from seed collected from different farms in Sweden and some seed from different European countries was compared. In 1907 aims and breeding goals were defined and Swedish forage crop breeding commenced (Julén, 1997). Early cultivation of red clover relied either on seed collected from the farm where it was to be grown or seed imported from Europe. Imported seed was less persistent than home grown seed. After cultivating a number of generations of local seed, enhanced local adaptation was often achieved. Cultivation of home grown seed resulted in several landraces, often cultivated at specific farms over long periods. These landraces were generally later heading, produced less re-growth and exhibited better winter survival the further north that they were grown. Many of these landraces were investigated for their cultivation value and the most valuable have been used for red clover breeding in Sweden.

The first Swedish red clover cultivar (cv.), 'Svalöfs Medeltidiga Rödklöver', was released in 1913 and was based on such a local collection from southern Sweden. When red clover cultivation increased during the 1920s and 30s, there were problems with clover rot and stem nematode (*Ditylenchus dipsaci*). In 1937, a cultivar with enhanced resistance to both pathogens, cv. Merkur, was released. Frandsen (1946) stated that it was impossible to find red clover plants that were immune to clover rot, but did demonstrate that natural selection in infected red clover material increased resistance to *S. trifoliorum* very considerably in the offspring compared to the unselected original material. Consequently, much of the early breeding for increased disease resistance was based on natural selection from infected fields. More recently, laboratory methods have been used alongside natural selection to breed for enhanced disease resistance to clover rot and root rot (Pulli *et al*., 1996). In the 1950s, a successful method to breed for resistance to the stem nematode was developed. Since then, all Swedish cultivars released onto the market have good resistance to stem nematodes (Andersson, 1986; Julén, 1997).

The early successes in breeding produced more disease resistant and winter hardy cultivars compared to the local collections or the imported cultivars, but no increase in total yield was achieved. In 1939, the first tetraploid red clover plants were produced in Sweden (Sjödin & Ellerström, 1986). The poor increase in dry matter yield, compared to diploid cultivars, in the early tetraploid breeding lines delayed the release of the first tetraploid cultivar until 1957. However, the poor seed set in the early tetraploid cultivars meant that they remained on the market for only a short time because of the restricted seed supply. In the mid 1970s, the first tetraploid cultivars that combined good seed set and enhanced yield were released. Later tetraploid cultivars, with better winter hardiness, have been released and since the 1990s tetraploid cultivars have been available for all regions of Sweden. (Julén, 1997).

Control of clover rot

Control methods for clover rot have included various cultivation practices, such as early sowing, ensuring good plant vigour in the autumn and deep ploughing after clover rot outbreaks. Foliar clipping in the autumn or winter has been tested in Crimson clover, *T. incarnatum*, (Pratt, 1991). It has been suggested that late cutting or grazing of clover stands may reduce the amount of plant material with the primary infection (Dillon Weston, Loveless & Taylor, 1946; Loveless, 1951) and that choosing an appropriate cutting time in relation to disease development can sometimes provide effective cultural control (Pratt, 1991). Dillon Weston, Loveless & Taylor (1946) concluded that, on land where clover rot has been troublesome, red clover should not be cultivated for at least eight years. The most common advice, however, has been to use resistant and well-adapted cultivars (Dillon Weston, Loveless & Taylor, 1946; Loveless, 1951).

It is possible to control clover rot by the use of chemical fungicides; some investigations have been performed to examine this in Sweden (Bengtsson, 1961; Vestman, 1971). Today, no such compound is legal in Sweden and, in addition, the use of fungicides is only ecologically and economically justifiable for production of valuable seed, not for forage production (Raynal *et al*., 1991).

Controlling diseases caused by *Sclerotinia* spp. through the use of antagonistic organisms has been investigated. The fungus *Coniothyrium minitans* was first described in 1947 by Campbell, who found it on a sclerotium of *S. sclerotiorum* (Whipps & Gerlagh, 1992). Since then this obligate mycoparasite has been found to occur naturally in soils around the world, with the exception of South America (Sandys-Winsch *et al*., 1993). Its action as a fungal biocontrol agent has been repeatedly verified, but the development of an effective and reliable commercial formula has been time consuming (de Vrije *et al*., 2001). One product, Contans®WG, is now available for the control of *S. sclerotiorum* and *S. minor*. It is only registered to be used against the former in Sweden, since the latter has not been reported from the Nordic countries (http://biologi.uio.no/bot/ascomycetes /Taxa/Sclerotinia.html; 5-Mar-2008).

Coniothyrium minitans has also been found in the field on sclerotia of *S. trifoliorum*; it has the ability to destroy sclerotia under certain circumstances (Tribe, 1957). In field experiments where sclerotia in retrievable nylon bags were treated with a pycnidial dust of *C minitans*, the antagonist successfully infected and reduced the viability of the sclerotia; this was more effective under sufficiently high moisture conditions (Turner & Tribe, 1975). It has also been found as a parasite on apothecia of this fungus under natural conditions (Wang & Vincelli, 1997). There are no previous studies of the antagonist *C. minitans*, the pathogen *S. trifoliorum* and the host red clover. To evaluate the possible action of the commercial product Contans®WG against the development of clover rot at cool climatic conditions, it was tested in a field experiment at Ås (III), a site where the most severe clover rot outbreak had been recorded (I).

Other mycoparasites on *S. trifoliorum* that reduce the number of sclerotia in soil have also been reported. These include *Episclerotium sclerotiorum*, previously known as *Mitrula sclerotiorum* (Röed, 1954; Ylimäki, 1968) and *Tricoderma viride* (Makkonen & Pohjakallio, 1960); these organisms have not been developed into commercial products.

According to Mishke (1997), Ayers & Adams (1981) reported that the mycoparasite *Sporidesmium sclerotivorum* infests *S. trifoliorum*. Adams (1989) found that *S. sclerotivorum* and *Teratosperma oligocladum* were able to infect and destroy the primary sclerotia of the plant pathogens *Sclerotinia sclerotiorum* and *S. minor* and were able to spread within natural soils to destroy previously uninfested sclerotia. *S. sclerotivorum* has a rather narrow range of hosts within the Sclerotiniaceae (Mishke, 1997), and del Rio, Martinson & Yang (2002) considered it to have potential as a biocontrol agent against *S. sclerotiorum* on soybean. The difficulties associated with producing commercial quantities of inoculum of this mycoparasite could pose a problem and no investigations into the suppression of *S. trifoliorum* by *Sporidesmium sclerotivorum* have been reported.

Aims

The aims of the studies presented in Paper I were: (i) to estimate the current extent of the occurrence of clover rot in Sweden; (ii) to evaluate the susceptibility of red clover cultivars to natural infection by clover rot in the field under different climatic conditions; (iii) to evaluate whether the performance of the cultivars under real agricultural regimes reflected resistance selection procedures by breeders or natural selection; and iv) to study the correlations between the susceptibility of these cultivars to clover rot estimated in laboratory tests (see Paper II) and their performance in field.

In Paper II, we examined whether, in red clover, the expression of resistance to clover rot is dependent on the level of aggressiveness of *S. trifoliorum* isolates. Studies of host–pathogen interactions between red clover cultivars and *S. trifoliorum* were performed in a controlled environment. To complement this study, the genetic diversity of *S. trifoliorum* isolates was examined (Study A), and the effects of ascospore and mycelial inocula on plants subjected to various hardening treatments were examined (Study B). Finally, a small screening investigation was conducted, to examine susceptibility to *S. trifoliorum* in other leguminous species (Study C).

One of the aims of the work described in Paper III was to investigate the effect of the biocontrol product Contans®WG, containing *Coniothyrium minitans*, in controlling *S. trifoliorum* under natural, cool conditions; this took the form of a single site field experiment. To complement this, the influence of temperature and incubation period on the viability of sclerotia of *S. trifoliorum* treated with *C. minitans* was studied in a controlled environment.

The aims of the work described in Paper IV were to document the occurrence and amount of root rot found at sites with different climatic conditions and to compare differences in red clover cultivar susceptibility to this disease.

Materials and methods

A summary of the research is presented in Table 1. The materials and methods used in Papers I–IV are briefly described below. The corresponding descriptions for the minor studies can be found within each study.

Table 1. *Summary of the research*.

Field studies		**Controlled environment studies**	
Paper I: Clover rot	15 red clover cultivars Five sites in Sweden Natural infection	Paper II:	20 red clover cultivars 20 isolates of *Sclerotinia trifoliorum* Mycelium-inoculated, hardened plants
Paper IV: Root rot	20 red clover cultivars Five sites in Sweden Natural infection	Study B:	Mycelium/ascospore inoculation and various hardening regimes
Study A:	*Sclerotinia trifoliorum* isolates derived from the field	Study C:	Nine species of forage legumes Mycelium-inoculated hardened plants
Paper III:	**Biocontrol of clover rot by Contans®WG, *Coniothyrium minitans*** One site, two trials grass-red clover ley and red clover in pure stand Various amendments Natural infection		Influence of temperature and incubation time on two *S. trifoliorum* isolates

Field experiments

The first experiments began in 1998. Twenty red clover cultivars (cvs), cultivated in pure stands, were used in field experiments at six sites exposed to a range of climatic conditions (Figure 9). The cvs used were adapted for growth in different regions of Sweden and were either medium–late or late flowering types. Both diploid and tetraploid cvs were included within each type. At each site ten cvs were included. Five northern types were common for all sites and the other five were of regional types. This design gave common cvs at sites Svalöv and Rådde (five southern and five northern), and at sites Lännäs, Ås and Öjebyn all 10 cvs were of northern types. Site Bjertorp had five intermediate cvs in addition to the five of northern types. All field experiments were conducted on agricultural land in Sweden; crop rotations were used at all sites and all had experienced clover rot infections in the past. The occurrence of clover rot in 15 red clover cvs, caused by natural infection of *S. trifoliorum*, was estimated at five of the sites in spring and autumn of the following two years (I). At all sites but Bjertorp clover rot infection occurred leading to registrations of disease in a total of 15 red clover cvs (I). At Ås in N. Sweden, plant death was substantial already after the first winter (see Figure 11).

To conclude the field experiments, red clover plants were dug up in order to estimate the occurrence of natural root rot infection at all sites but Ås where there

were not many plants left (Figure 9). The root rot survey thus included all 20 red clover cvs. A root rot disease index, based on internal and external damage, was calculated according to Rufelt (1979) for all red clover cvs grown at these five sites (IV).

In order to get isolates of various origins, *Sclerotinia trifoliorum* sclerotia or apothecia were collected in field from the field experimental sites, Röbäcksdalen and two additional sites (Figure 9). Single isolates were obtained from sclerotia or ascospores, using various methods adapted from the work of Björling (1942). After plating on agar media (Bång, 1989) in Petri dishes, some were used as single isolates in the controlled environment tests and some in the additional studies (II, III, Studies A, B and C).

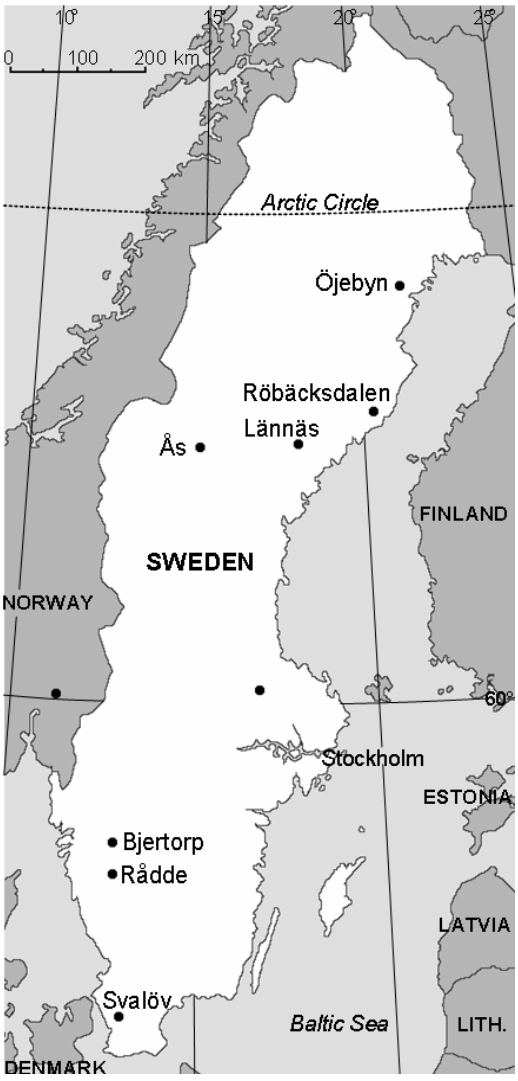

Figure 9 Field experiments were conducted at six sites in Sweden. Results from Svalöv, Rådde, Ås, Lännäs and Öjebyn were used in the clover rot investigations (I). The effects of Contans®WG treatment were studied at Ås (III). Red clover plants cultivated at Svalöv, Rådde, Bjertorp, Lännäs and Öjebyn were used in the root rot study (IV). The controlled environment tests (II) were performed at Röbäcksdalen. Additional *S. trifoliorum* sclerotia and apothecia were also collected from the two remaining sites (unlabelled dots).

Controlled environment tests

The same 20 red clover cvs as examined in the field experiments were used in these studies. All red clover seeds were inoculated with *Rhizobium leguminosarum* bv. *trifolii* strains 2080 and 2085 from Baljväxtlaboratoriet, Uppsala, Sweden, when germinated. Plants were cultivated in a greenhouse for eight weeks in seed trays containing a commercial potting mixture before being subjected to hardening and inoculation procedures in controlled environment. G. Vestman and SWAB (unpublished data) developed the cold-hardening treatment of plants used in these tests. This treatment has been used by SWAB at Röbäcksdalen for several years during breeding for enhanced clover rot resistance in red clover.

Dry grain inocula for mycelial inoculation of *S. trifoliorum* were produced (using a technique adapted from Kreitlow, 1951) for inoculation studies. When used, this sclerotia/mycelia/grain mixture was coarsely ground and evenly applied onto the plants.

Materials and methods used in the minor studies A, B and C are found below.

Biological control experiments

Field experiment

*S*ingle site field experiments were established at Ås, close to the mountainous area of central Sweden (Figure 9). The biocontrol compound Contans®WG was applied, after the first cut of the year, to an established grass–red clover ley, known to suffer from clover rot infection. A pure stand red clover experiment was also established, using two red clover cvs. Contans®WG was applied either before seeding or to young seedlings. Both experiments included untreated control plots. The application rates and techniques used were in accordance with the recommendations of the supplier. In the autumn, apothecia were counted within sub-quadrats of the plots. Clover rot occurrence was estimated in the spring of the following year (III).

Controlled environment test

Sclerotia from two different *S. trifoliorum* isolates were treated with Contans®WG, added to moist soil in Petri dishes. Dishes were incubated at temperatures ranging from 5 to 15 °C, for three to seven weeks. The viability of sclerotia was recorded using a detached leaf test (adapted from Delclos, Mousset-Déclas & Raynal, 1997).

Additional studies of *Sclerotinia trifoliorum*

Study A: A study of the mycelial compatibility of some Swedish *Sclerotinia trifoliorum* isolates.

In earlier studies, some red clover cultivars were found to exhibit clear differences in their resistance to clover rot depending on growth location. A number of authors (Björling, 1942; Frandsen, 1946; Vestad 1955; Dixon & Doodson, 1974) discussed the occurrence of local strains of *S. trifoliorum*, which differ in their ability to cause disease depending on local conditions. Later, Pratt & Rowe (1995) and Halimi, Rowe & Pratt (1998) concluded that different *S. trifoliorum* isolates, originating from different regions, differed in their ability to infect some alfalfa (*Medicago sativa*) cultivars; they were not, however, considered to constitute different pathological races. Since there was no consensus about the occurrence of local strains, this study was performed to give an indication of the genetic diversity of *S. trifoliorum* in Sweden.

Materials and methods

To obtain single isolates of *S. trifoliorum*, different methods (adopted from Björling, 1942) were used. In total, 12 isolates originating from apothecia and 241 from sclerotia were investigated.

In autumn 1998, apothecia were collected from the experimental sites at Hamre, Ås and Öjebyn. For further information on these sites, see Paper I. Three isolates each were obtained from Hamre and Ås, using an adapted version of the method presented by Björling (1942) where red clover leaves and stems are used as the initial growth media. Clover leaves, with approximately 5 cm petioles were collected. Petioles were surface-sterilised for 3 min in 2% sodium hypochlorite (*aq*.) and rinsed for 5 min in tap water. The petioles were inserted, through a small opening in the cap, into small glass jars containing sterile glucose agar medium (Bång, 1989). Sterilised cotton was used to seal the openings. A small piece of an apothecium was placed on the leaf and released spores grew through the petiol onto the agar media in the jars. The mycelium was collected from the agar for further growth on fresh agar medium in 9-cm Petri dishes. Six isolates were collected from Öjebyn; these were derived from single apothecia, which were macerated in water and plated onto agar medium in Petri dishes.

Sclerotia were collected from the experimental sites and from other sites in Sweden. In addition, one isolate was prepared from a Norwegian sclerotium. Isolates were prepared using two methods: i) the direct method described in Paper II, where surface sterilised sclerotia are placed on agar medium; or ii) by the indirect method described above, where unsterilised sclerotia were placed on clover leaf surfaces. Four isolates were old and originated from those used by Vestman (1993).

Sclerotia were collected in 1999 from the field experiment sites (I) to investigate the genetic diversity within a field. The total area of each experiment was around 900 m^2. Sixty-one of the sclerotial isolates derived from 21 plots, at the Svalöv field experiment site. One or two sclerotia were collected from each plot and each of the 33 sclerotia produced one or two isolates. Fifty-nine isolates were obtained from 20 plots at Hamre. Thirty-eight isolates were obtained from 16 plots at Ås. Twenty nine isolates were obtained from 17 plots at Öjebyn. All these were used to study the variation between isolates found at a single site. Each plot was approximately 12 m^2.

Mycelial compatibility was tested using a method described by Björling (1942). If two isolates of *S. trifoliorum* are genotypically different, and thus exhibit mycelial incompatibility, a reaction line will form between mycelia of the two when grown on agar medium (Björling, 1942), (Figure 10).

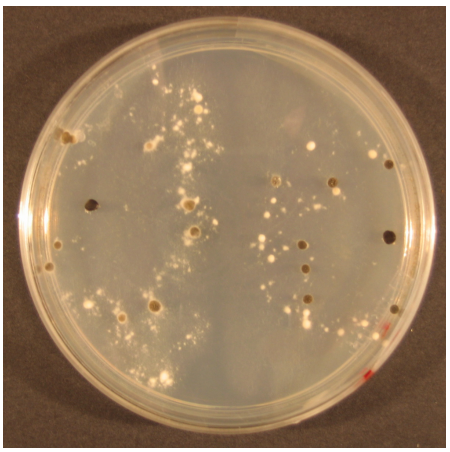

Figure 10. Two isolates of *Sclerotinia trifoliorum* plated onto agar in a Petri dish, exhibiting a typical incompatibility reaction.

Sclerotia or small mycelial plugs were plated onto the same sterile glucose agar medium in 9-cm Petri dishes. The agar medium also contained 1.5 ml l^{-1} of red food colouring (Kohn, Carbone & Anderson, 1990); this increased the visibility of reaction lines.

Results

Ascospore isolates

In two cases, the ascospores from a single site exhibited no mycelial incompatibility: the three isolates derived from two apothecia collected at Hamre and the six isolates derived from a single apothecium collected at Öjebyn. This is in agreement with Björling (1942), who stated that no reaction lines were found in three cases: i) if single spore isolates derived from different apothecia, all from the same isolate, were tested against each other; ii) if a single spore isolate from an apothecium was tested against mycelia from the sclerotium from which the apothecium was derived; and iii) between 'fertile' and 'sterile' isolates derived from large and small spores from the same ascus. At Hamre, the two apothecia must have had the same origin, thus accounting for the absence of reaction lines. In addition, the two or six isolates from the same apothecium should not have

produced reaction lines if they were derived from small or large ascospores from a single ascus. Björling's statements are correct as long as no mating between isolates has occurred. The ascospore isolates from Ås, however, all exhibited mycelial incompatibility, even though two of them originated from a single apothecium. This would be the case if mating between isolates had occurred at this site. This contention is supported by the work of Rhenstrom & Free (1993), who found that isolates generated from progeny, after mating, generally differed genetically from each other.

Sclerotial isolates

At Svalöv mycelial incompatibility was found in four out of 12 cases for isolates originating from the same trial plot, *i.e.* between two sclerotia collected within the a single plot. When 69 mycelial combinations involving isolates from different plots were tested, reaction lines were formed in all cases. The corresponding results from Hamre were that 35 out of 68 possible combinations within plots had no reaction lines between isolates. All 78 combinations involving isolates from different plots produced distinct reaction lines. At Ås, four out of 31 combinations within plots had no reaction line. In addition, three combinations of the total of 38 tests involving different plots were compatible. These three combinations were all between isolates collected from plots located next to each other. Isolates from Öjebyn showed the highest degree of mycelial incompatibility. Only one combination of isolates found within a plot, out of 14 tested, showed compatibility; the 54 combinations involving different plots did not. The results from this investigation at the experimental sites demonstrated that there is extensive genotypic variation in the field. It also showed that genetically related isolates occurred within a radius of approximately 10 m.

When fifty-one isolates of *S. trifoliorum* collected from several locations in Sweden were examined in all possible combinations (2601) for their mycelial vegetative incompatibility, only a few (26) combinations were compatible. Some of the isolates in this study originated from the apothecia discussed above and thus three of the compatible reactions were associated with the isolates from Hamre. Most of the other compatible isolates originated from a single site (Bjertorp). Only ten combinations of isolates collected from different sites were compatible. Some of these originated from very distant experimental sites (>1000 km).

The remaining isolates obtained from sclerotia were not tested for all possible combinations, but they were all incompatible in the combinations that were tested. Incompatibility was, thus, found within each location and each geographic area, irrespective of the red clover cultivar from which the original sclerotia had been collected. Consequently, the same level of diversity was found between different locations within a region as between regions.

Conclusion

These investigations showed that single isolates with a unique mycelium are easily found; this is in accordance with the findings of Rhenstrom & Free (1993). They concluded that at least three mycelial incompatibility genes segregate in crosses

between isolates; this explains why so many different incompatible isolates exist. The genetically different isolates derived from one apothecium from Ås, reveal that genetic recombination occurs. This, combined with finding so many unique isolates within each site, indicates that local strains of *S. trifoliorum* probably do not exist in Sweden, contrary to the suggestion of Björling (1942).

Study B: Effects of different hardening regimes and inoculation sources on clover rot infection of red clover plants.

As a complement to the experiments performed in Paper II, a small test using different hardening regimes and comparisons between ascospore and mycelial inoculations of plants was conducted. Several studies (Dijkstra, 1964; Raynal, 1981; Marum, Smith & Grau, 1994) have compared ascospores and mycelium as inoculation sources and, generally, only slight differences have been found for red clover plant survival or disease severity. Most of these studies, however, were performed on unhardened seedlings. This experiment was undertaken to study the impact of inoculum source under various hardening regimes.

Materials and methods

The red clover cv. Björn was subjected to eight different treatments, each replicated twice. Inoculations were performed using either ground sclerotia (II) or an ascospore suspension of the *S. trifoliorum* isolate no. 5 (II). The ascospore suspension was prepared according to Marum, Smith & Grau (1994). Plants were grown and incubated in trays containing a maximum of 98 plants, according to the methods described in Paper II. Light and temperature conditions during hardening were as described in Paper II, but the duration of the procedures varied in this study. The combinations of plant treatments were: unhardened and uninoculated (control), unhardened and inoculated with mycelium (ground sclerotia) or ascospores, pre-hardened for one week prior to ascospore infestation, pre-hardened for one week and then hardened for one week prior to ascospore inoculation, fully hardened as described in Paper II prior to mycelial or ascospore inoculation and finally plants were cut prior to hardening and inoculated by ascospores after the full hardening period (see Table 2). Statistical calculations were performed in NCSS using untransformed survival percentages; the control data were not included. Differences between means were determined using Fisher's protected LSD method.

Results

Unfortunately, one of the control trays was lost, but 91 % of the plants in the other replicate survived. Mycelial inoculation caused more plant death than did ascospore inoculation under the same hardening treatments (Table 2). Unhardened plants inoculated with ascospores had a higher survival rate than plants with incomplete hardening, but full hardening resulted in the highest plant survival. The differences, however, were not significant. For those plants that were fully

hardened, the ones that were trimmed before hardening suffered significantly more than those trimmed afterwards.

Table 2. *Relative numbers of surviving red clover plants (cv. Björn) after different hardening regimes prior to mycelial or ascospore inoculation with the same* Sclerotinia trifoliorum *isolate. Mean values of two replicates (only one for the control), each containing data from 98 plants.*

Hardening regime	Inoculation source	Surviving plants, %
Unhardened (control)	uninoculated	91.0
Unhardened	mycelium	0
Unhardened	ascospore	17.2
Prehardened	ascospore	7.1
Prehardened + 1 week hardening	ascospore	11.5
Full hardening	mycelium	2.2
Full hardening	ascospore	24.3
Trimmed prior to full hardening	ascospore	3.2
LSD $_{\alpha = 0.07}$		16.2

Conclusion

This pilot study showed that uninoculated plants were unharmed by the low temperature and darkness during the incubation period, indicating that a rapid shift in growth conditions alone is not lethal to red clover plants. The mycelial inoculum caused more plant death due to clover rot than the ascospore inoculum, irrespective of whether the plants were hardened or not. The wounds inflicted by trimming before hardening may have provided an entrance for the pathogen, and disturbance of the plants at this stage seems to have made them more susceptible to infection than those that were trimmed after hardening.

Study C: *Sclerotinia trifoliorum* on different host species.

Data on clover rot susceptibility of other legumes with potential to be cultivated in Sweden is limited. Some studies concerning the difference in susceptibility among forage legumes have been undertaken in Denmark (Frandsen, 1946) and the USA (Kreitlow, 1949). A small screening investigation was therefore undertaken, involving nine species of forage legumes.

Materials and methods

The clover rot resistance test method described in Paper II was used to study the interactions between two isolates of *S. trifoliorum* and, in total, nine species of forage legumes. Three clover species, red clover (*T. pratense*), white clover (*T. repens*), and alsike clover (T. *hybridum*), were compared, along with alfalfa (*Medicago sativa*), yellow sweetclover (*Melilotus officinalis*), bird's-foot trefoil (*Lotus corniculatus*), large bird's-foot-trefoil (*Lotus pedunculatus*), sainfoin (*Onobrychis viciifolia*) and goat's-rue (*Galega orientalis*). Since susceptibility to clover rot varies between cultivars of the same species, more than one cultivar of

most species were tested. A wild population of *T. repens*, originating from Ås were tested since white clover is found less susceptible than red clover at this site (Per-Erik Nemby, personal comunication).

Mycelial inoculation with two *S. trifoliorum* isolates, no. 5 and 16 (see Paper II), was used in this controlled environment experiment. All species and cultivars were treated the same way and plant material was grown, hardened and incubated according to the cultivation method described in Paper II, in trays containing a maximum of 98 plants. After two months incubation in darkness at low temperature, plant survival was recorded; plants were first allowed to recover in a greenhouse. Statistical calculations were performed in NCSS using untransformed survival percentages. Differences between means were determined using Fisher's protected LSD method.

Results

There were significant differences among the *T. repens* and *L. corniculatus* populations included in this study (Table 3). The most resistant cultivars of these species survived better than the two *T. pratense* cultivars, which in turn exhibited similar survival levels to *M. sativa*. The lowest survival occurred in *O. viciifolia* and *M. officinalis*.

Table 3. *Percent surviving plants the greenhouse experiment. Fourteen populations of forage legumes were tested for resistance to two isolates of* Sclerotinia trifoliorum *originating from north-eastern (Isolate 5) and south-western (Isolate 16) Sweden. The numbers are means of four replicates (two per isolate).*

Species (cultivar/ population)	Percent surviving plants
Galega orientalis	61.8
Lotus corniculatus (Leo)	62.4
Lotus corniculatus (GA1)	36.6
Lotus pedunculatus	21.3
Medicago sativa (Julus)	19.5
Medicago sativa (Pondus)	18.4
Melilotus officinalis	5.4
Onobrychis viciifolia	8.3
Trifolium hybridum (Frida)	29.8
Trifolium pratense (Björn)	14.2
Trifolium pratense (Britta)	17.0
Trifolium repens (Undrom)	65.6
Trifolium repens (Sonja)	45.8
Trifolium repens (Ås)	37.8
LSD $_{\alpha = 0.05}$	21.6

Conclusion

The experiment showed that all the species studied were susceptible to clover rot, but the degree varied; this is in agreement with both Frandsen (1946) and Kreitlow (1949). In some species, there were significant differences between populations. Red clover was among the most susceptible species.

Results and discussion

Clover rot was found throughout the country (I), showing that this disease is still a major problem associated with red clover cultivation, despite long-term selection for disease resistance when breeding new cultivars. The losses due to clover rot ranged from 0 to 87% at the experimental sites in the first winter after sowing. All red clover cultivars included in the field experiments were susceptible to *S. trifoliorum*, but the level of susceptibility varied; this was confirmed in the controlled environment tests (II).

Clover rot had been reported at all the sites studied, but none of them had an over-wintering legume crop cultivated immediately prior to the investigation (I). At Ås, where the most severe clover rot outbreak was recorded (Figure 11), legumes had not been cultivated for eight years. This confirms the observations of Dillon Weston, Loveless & Taylor (1946), that severe outbreaks of clover rot can occur on land after long periods when a host has not been cultivated. However, at Ås, white clover (*T. repens*) is a common weed and this might have helped to maintain the viability of sclerotia in soil. In support of this assumption, study C revealed that this population of *T. repens* was the most susceptible one of the three tested, indicating its suitability as a host for the pathogen.

Figure 11. Plant death caused by natural *S. trifoliorum* infection at Ås during the first winter after seeding. Red clover was grown in pure stand plots surrounded by grass.

Among the other sites with clover rot infection, the magnitude was not correlated with weather conditions during the first winter. Mortality due to clover rot in the five cultivars common to all field experiments, was not significantly different at the Svalöv and Lännäs sites after the first winter (I), despite different weather conditions during this period.

Tetraploid forms of red clover were clearly more resistant to clover rot than diploids in field experiments in northern Sweden (I; summarised in Figure 12). This relationship has been reported previously in several studies (Vestad, 1955, 1960; Dijkstra, 1964; Raynal, 1985; Arseniuk, 1989).

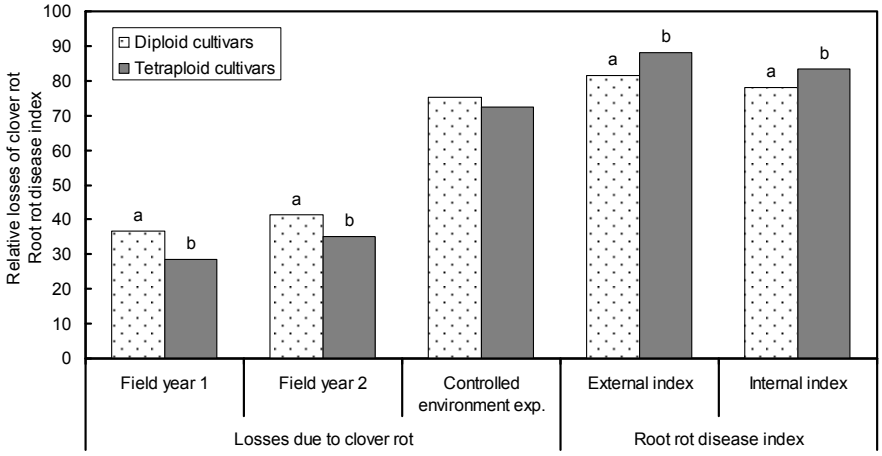

Figure 12. Relative losses in diploid and tetraploid red clover cultivars due to clover rot in field experiments in N Sweden (means of 10 cultivars at three sites) and in a controlled environment (means of the same cultivars); and root rot disease index of the same cultivars recorded from two field experiments in N Sweden. Different lower case letters above the bars indicate significant differences between means within data pairs. Data based on results from Papers I, II, IV.

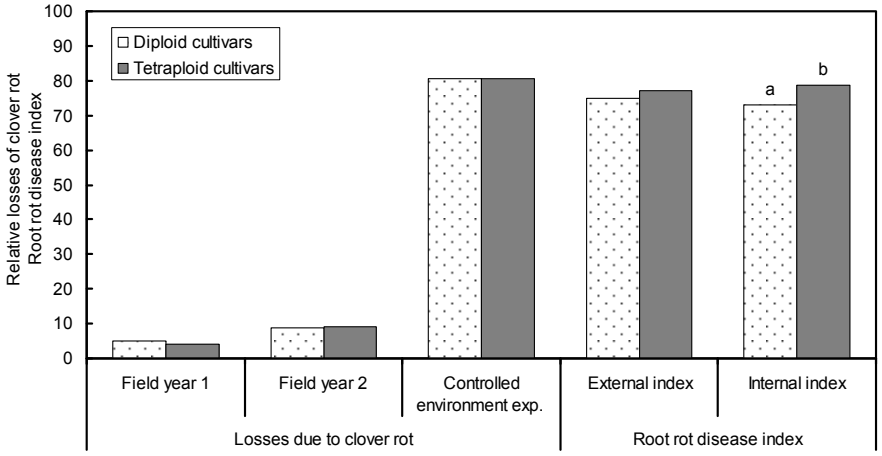

Figure 13. Relative mortality of diploid and tetraploid red clover cultivars due to clover rot in two field experiments in S Sweden (means of 10 cultivars); mortality of 20 different red clover cultivars in a controlled environment; and root rot disease index recorded in field experiments in S Sweden (mean values from three sites and 15 cultivars). Different lower case letters above the bars indicate significant differences between means within data pairs. Data based on results from Papers I, II, IV.

In southern Sweden, the results from field experiments differed between sites and no general difference between the ploidy types was found (I; Figure 13). In addition, the results from the controlled environment tests (II) revealed no overall difference between ploidy types (Figures 12, 13).

Vestad (1960) and Arseniuk (1989) both studied the relationship between the susceptibility of various ploidy types in detail. In general, they did not find tetraploids to be more resistant than diploids. However, in a ploidy pair, the autotetraploid was always found to be more resistant than its ancestral diploid. This implies that differences in resistance between cultivars of different ploidy types, excluding autotetraploid couples, may be due to various stochastic factors including the choice of cultivars studied. This could explain the seemingly contradictory results at sites in N and S Sweden involving different cultivars. The true difference in response to *S. trifoliorum* infection between diploids and tetraploids can, consequently, only be determined when auto-breed cultivars are studied (Arseniuk, 1989). Cultivars Bjursele (2x) and Betty (4x) constitute such a pair, and results from field experiments agree with the general contention that tetraploids are less susceptible to clover rot infection (Figure 14). However, in a single site field experiment (III) another pair, cvs Jesper (2x) and SW Torun (4x), exhibited no significant differences in mortality due to clover rot in untreated plots (Figure 15). Also, in the controlled environment experiment (II), the diploid cv. Bjursele had significantly lower mortality than the tetraploid cv. Betty (Figure 14).

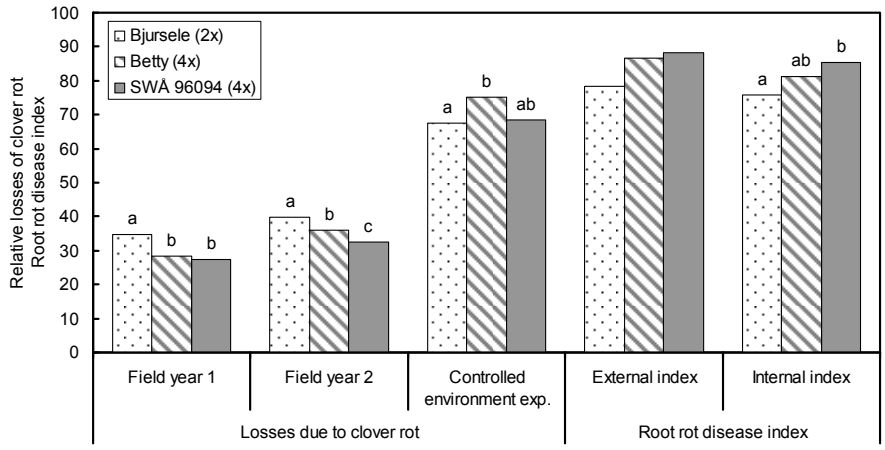

Figure 14. Relative losses due to clover rot and disease index of root rot in the three red clover cultivars, Bjursele, Betty and SWÅ 96094, which constitute a breeding/selection line. Different lower case letters above the bars indicate significant differences between means within data groups. Data based on results from Papers I, II, IV.

The lack of significant differences in susceptibility between cultivars of different ploidy levels in laboratory tests (II) might be because conditions for clover rot infection were optimised. Other authors (Dijkstra, 1964; Raynal, 1985; Arseniuk, 1989) have also recorded non-significant differences between diploids and tetraploids under such conditions. In addition, the two cultivars included in the

experiment with the biological control (III), also an autoploidy pair, were attacked equally in untreated plots (Figure 15). The level of disease was very severe at this site; this might, based on the suggestion above, explain the absence of superior resistance in the tetraploid cv. SW Torun.

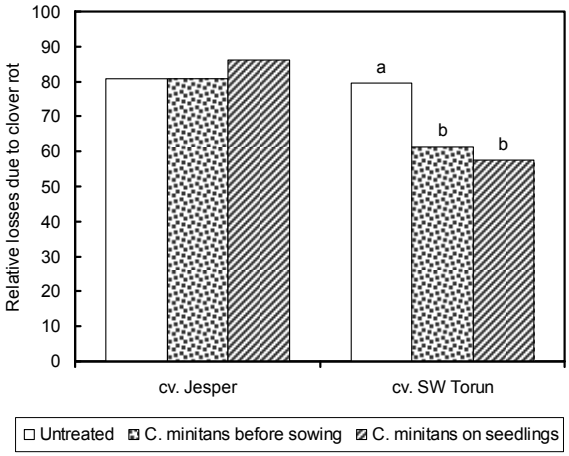

Figure 15. Relative losses due to clover rot after the first winter, involving a devastating attack of clover rot, in two red clover cultivars: the tetraploid SW Torun and its diploid ancestor Jesper; treatments included application of the antagonist *Coniothyrium minitans* on two occasions, as indicated. Different lower case letters above bars indicate significant differences between means within data groups. Results from Paper III.

In the controlled environment test method used in Paper II to study the interactions between red clover cultivars and different isolates of *S. trifoliorum*, a mycelial inoculation method modified after Kreitlow (1951) was used. Under natural conditions, ascospore infection is thought to be the predominant source of primary inoculum (Dijkstra, 1966). However, the initial ascospore infection on a plant's surface may not progress and there may be no further spread of infection. The fungal spread within a plant is, in fact, the result of mycelial growth after the plants have been weakened, possibly by frost or snowfall (Dijkstra, 1966). Mycelial inoculation of cold-hardened and damaged (trimmed) plant material might, therefore, resemble the later stage of infection. Significant correlations, with coefficients between 0.69 and 0.74 for different sites, were found between the controlled environment tests of resistance and the survival of plants in field experiments (I). This demonstrates that the inoculation method involving the use of ground sclerotia, is suitable to assess this plant–pathogen relationship.

The hardening procedures were found to strongly influence the survival of red clover plants in Study B and Paper II confirming the findings of other authors (Hesselman, 1962; Arseniuk, 1989). In addition, survival of first year alfalfa plants has been found to be better the longer they are exposed to hardening conditions in the field, prior to inoculation with the low-temperature basidiomycete fungus causing winter crown rot (Hwang & Gaudet, 1995). This supports the suggestion that the hardening process is a prerequisite for expression of resistance against *S.*

trifoliorum, and that resistance linked changes, which differed amongst the cultivars, occurred in the plants during the hardening process. In addition, non-winter-hardy clover cultivars are generally considered to be more susceptible to clover rot than late and winter-hardy ones (this is common knowledge, and there is empirical data; Dijkstra, 1964). However, the cultivar Vå 092001, originating from an area of Norway where clover rot is not prevalent, is very hardy but was found to be very susceptible to clover rot (I, II), indicating that hardiness and resistance are not necessarily linked.

In addition to the ploidy of red clover cultivars, the flowering type was found to influence disease susceptibility (I, II, IV), the late-flowering ones being less attacked by both clover rot and root rot (Figure 16). This may be a consequence of greater hardiness associated with the late-flowering types, see above.

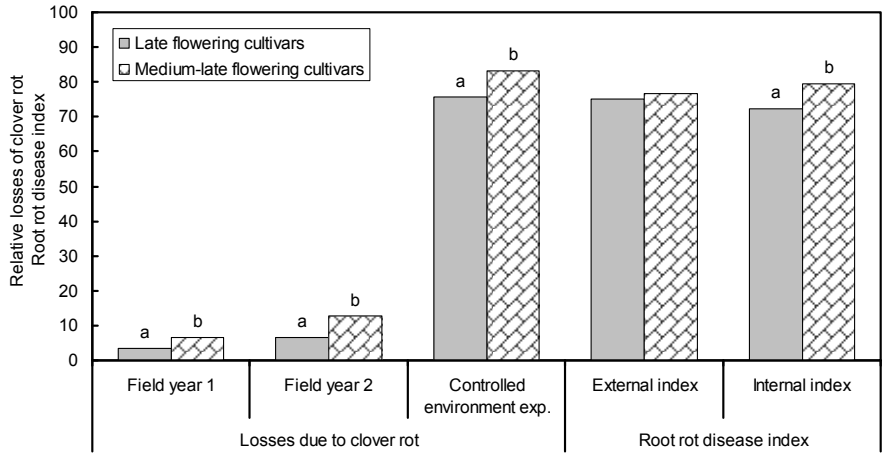

Figure 16. Mortality due to clover rot in late and medium–late flowering red clover cultivars in two field experiments in S Sweden (means of 10 cultivars); mortality of 20 different red clover cultivars in a controlled environment; and root rot disease index observed in field experiments in S Sweden (mean values from three sites and 15 cultivars). Different lower case letters above the bars indicate significant differences between means within data pairs. Data based on results from Papers I, II, IV.

In Study B, using different inoculation sources in a controlled environment, the mycelial inocula produced much more severe infections than the ascospore inocula, irrespective of whether the plants were cold-hardened or not (Table 2). The results from this study indicate that ascospore inoculation might not be the best method to obtain an optimal correlation with the survival recorded under field conditions involving very severe infection, such as those encountered at Ås (I, III). Marum, Smith & Grau (1994) found only slight differences in red clover plant survival or disease severity when they compared ascospores and mycelium as inoculation sources. However, they used unhardened and undamaged two-week old seedlings that were incubated at 15 °C with a 12 h day-length for 10 to 14 days. These conditions do not resemble natural conditions in the field. Plants are usually older than two weeks when they are exposed to *S. trifoliorum* ascospores

in the field and, since plant resistance increases with plant age, see below, their findings are not comparable with the results in Study B. Dijkstra (1964) and Raynal (1981) found no evidence that ascospore inoculation was more valuable in determining resistance than the customary mycelial infection. Taken together, these results indicate that the commonly used mycelial inoculation method, which is simpler to perform, is adequate.

The results of Paper I, did not suggest any differences between selection procedures for increasing disease resistance to clover rot. Cultivars selected to have enhanced clover rot resistance after natural infections in the field and cultivars selected after artificial inoculation under controlled conditions both had the same level of mortality due to clover rot in the field in S Sweden (I). The breeding/selection line consisting of cvs Bjursele (2x), Betty (4x) and SWÅ 96094 (4x) demonstrates that breeding red clover can increase resistance to clover rot in the field (Figure 14), as described by several authors (*e.g.* Frandsen, 1946; Hesselman, 1962; Arseniuk 1989).

Great genetic variation was found amongst the *S. trifoliorum* isolates in Study A. Twenty of these isolates were used to study whether plant mortality varied accordingly (II). The 20 isolates differed significantly with respect to their aggressiveness and, as a group, the isolates originating from N Sweden generally caused higher plant mortality than those from S Sweden, even though significant differences were also found within each group. In addition, for the isolates from S Sweden, significant differences in aggressiveness were found among isolates from the same field at three out of the four study sites. The large genetic variation found in *S. trifoliorum* (II, Study A), indicates that local strains do not exist in Sweden. These results are in agreement with the findings of Pratt & Rowe (1995) and Halimi, Rowe & Pratt (1998), but do not support the assumptions of Björling (1942) relating to the existence of different pathological races (see above).

The implication that specific regional or pathological races of *S. trifoliorum* exists, probably depend on the lack of consistent results found in field experiments regarding the susceptibility among red clover cultivars (records in *e.g.* Frandsen, 1946; Vestad 1955; Dixon & Doodson, 1974). Halimi, Rowe & Pratt (1998), studied the interaction between *S. trifoliorum* and its host plant (alfalfa) and found that the response to *S. trifoliorum* inoculation depended more on the genotype of the plant than on the genotype of the fungal isolate. This indicates that the physiological response of red clover plants to local growth conditions are more decisive for the outcome of the host-pathogen relationship than the genotypes of *S. trifoliorum* found at that site.

The experiment described in Paper II, using red clover cultivars, shows that there can be great variation in resistance to clover rot within a species. In Study C, the same level of variation was found among cultivars of other species (Table 3). Therefore, one should be very careful when evaluating results from experiments based on limited material of various species. However, the very high mortality of yellow sweetclover (*Melilotus officinalis*), and sainfoin (*Onobrychis viciifolia*)

indicates that breeding for clover rot resistance in these species might be a difficult task.

The most extensive damage due to clover rot was found at Ås (see Figure 11). In Paper III, experiments conducted in the field, that these records were collected from, showed that *Coniothyrium minitans* was able to reduce mortality in cv. SW Torun but not in cv. Jesper compared to the untreated control (Figure 15). The reason for the difference in response to treatment between the two cultivars is unclear.

In the field experiments at the same site, in which *C. minitans* was applied to an established grass-red clover ley, no reduction in mortality was recorded in the treated compared to the untreated plots. However even in the first autumn, the number of apothecia found was somewhat smaller in the treated plots and a year later significantly fewer groups of apothecia were found in the treated compared to the untreated plots. This indicates that the antagonist can survive and multiply even under very harsh climatic conditions. This is supported by results from the laboratory experiment, where *C. minitans* was able to significantly reduce the viability of *S. trifoliorum* sclerotia at all temperatures tested. The greatest reduction in viability was obtained after a treatment period of seven weeks in one of the tested *S. trifoliorum* isolates; the pattern was not as distinct for the other isolate tested. The results indicate that *C. minitans* might be a suitable biocontrol agent for clover rot in Sweden, although the compound tested, Contans®WG, was originally designed to control *S. sclerotiorum* and *S. minor*.

Root rot symptoms were found throughout the country and in almost all plants examined (IV). Root rot indices varied between 65.0 and 83.6 for internal symptoms and between 71.6 and 87.3 for external symptoms, indicating that roots were very severely attacked. (Figures 12, 13, 14, 16). The results clearly show that root rot is an important factor affecting the lack of persistence of red clover, as has been demonstrated in studies in S Sweden (Lager, 2002; Wallenhammar *et al*., 2006). In contrast to clover rot, root rot was more severe in tetraploid cultivars than in diploids (Figures 12, 13). This is difficult to explain, but might be due to the plant types having different physiologies (Sjödin & Ellerström, 1986). The resistance mechanisms associated with the two diseases are evidently not the same; this is not surprising considering their different natures. This, of course, complicates the breeding processes necessary to increase the persistence of red clover

Conclusions

Clover rot and root rot are both prevalent in Sweden. They cause considerable damage to red clover and constitute a great threat to its persistence.

Tetraploid red clover cultivars are generally more resistant to clover rot but more susceptible to root rot than diploid cultivars under field conditions.

Late-flowering types of red clover are more resistant to both diseases than medium-late flowering ones.

The clover rot pathogen, *Sclerotinia trifoliorum*, consists of different genotypes with different abilities to cause disease.

Cold treatment was a necessary prerequisite for the expression of resistance to *S. trifoliorum* in red clover cultivars.

Clover rot originating from natural soil infestation of *S. trifoliorum* was controlled by *Coniothyrium minitans* under cool climatic conditions.

Concluding remarks

The laboratory test methods for screening of clover rot resistance of plants described in this thesis are very time-consuming and require a large amount of space; they are, however conclusive. Other methods, such as detached leaf tests, could have been used for testing differences in susceptibility among red clover cultivars to *S. trifoliorum*. However, cultivars consist of populations of individuals so each cultivar had to be represented by several individuals to make results more reliable. In addition, the detached leaf tests require as many working hours. The red clover plants used herein were close in age to those which are likely to be exposed to *S. trifoliorum* in real agricultural situations. The hardening procedure used had been designed by G. Vestman and SWAB to produce the best results (unpublished data), and was therefore not subjected to any further studies.

Certain types of studies can only be performed in the field and there are seldom any shortcuts. The fruiting bodies (apothecia) of *S. trifoliorum* are tiny, the resting bodies (sclerotia) even smaller and the primary infections on red clover leaves are only visible when plants are examined closely. Therefore, any researcher requires a lot of patience, a strong back, good eyesight and appropriate clothing (warm, wind- and waterproof); an appropriate pair of tweezers is also useful.

Raynal (1985) described the nature of counting apothecia in field as follows: *"Each episode of counting, in a 50 m^2 area, took about 3 to 4 hours of work in a very uncomfortable position, under rarely pleasant weather conditions."* So, I am in good company and share my experiences with other researchers who performed many of their experiments several decades ago; I am not the only one who has spent time crawling around on my knees for several hours in miserable weather to collect good scientific data (Figure 17).

Figure 17. Data collection on a rare day with clear skies. Photo: Per-Erik Nemby.

The research described in Paper IV on the composition of fungi involved in the root rot complex, was conducted under severe financial constraints. More time, money and better methods for identifying the fungi involved (PCR, DNA sequencing, T-RFLP or similar) would certainly have provided a better picture. Laboratory tests on mature red clover plants could have revealed some differences in pathogenecity among isolated fungi and helped to explain which of them is most likely to cause the initial symptoms on roots.

The two diseases studied in this thesis are the ones that have the grates impact on red clover persistence in Sweden. However, several other organisms can attack red clover including other fungi, bacteria, mycoplasma, viruses, slugs, insects, mites and nematodes. Besides the stem nematode (*Ditylenchus dipsaci*) mentioned above, most of them have no or minor effect on the persistence of red clover in leys. Pathogens that attack red clover during the vegetations period mostly affect growth vigour, herbage quality and sometimes the total dry matter yield. As red clover in ley is frequently cut, and the possible infected tissues then are removed from the field many of these diseases are most important in red clover seed production (Frame, Charlton & Laidlaw, 1998).

Future research

In addition to using resistant cultivars, significantly reducing the amount of soil born inocula, *i.e.* the sclerotia, of *S. trifoliorum*, through the use of the commercially available biocontrol compound Contans®WG has the potential to enhance persistency in red clover stands. The initial studies described in Paper III need to be continued in order to find an appropriate, cost-effective way of using this product to control clover rot.

Biofumigation using biocidal compounds released by certain crops used in crop rotations and as green manures to suppress soilborne pathogens may also provide a method for controlling clover pathogens abundant in soil. Plants belonging to the *Brassicaceae* contain sulphurous compounds, glucosinolates, which are converted, through hydrolysis, to volatile isothiocyanates; this process is known as "biofumigation" (Sarwar *et al*., 1998). Biofumigation is effective against several different soil borne plant pathogenic fungi with different host ranges; this increases the benefit of including appropriate species in the crop rotation (Larkin & Griffin, 2007). Suppressed growth of *S. sclerotiorum* in the presence of *Brassica oleracea* (Li *et al*., 2006) and *Brassica juncea* (Larkin & Griffin, 2007) has been reported from laboratory studies. Larkin & Griffin (2007) also found reduced mycelial growth in some *Fusarium* spp. Further studies are needed to elucidate the use of macerated *Brassicaceae* plants as a biocontrol for clover rot and root rot pathogens. It is important to determine the extent to which volatiles may reduce the viability of *S. trifoliorum* sclerotia in soil, as well as examining their ability to reduce or prevent mycelial growth in the field. Since biofumigation causes changes in the microbial community structure, care must be taken to prevent disturbance of the microbial symbionts, including *Rhizobium leguminosarum* bv. *Trifolii*, in order to ensure good red clover persistence.

Both of the biocontrol methods mentioned above are suitable for organic systems but could, equally, be applied to conventional farming.

One of the aims of these studies, was to evaluate the methods used for selection of more resistant cultivars. The results showed that the common selection procedure – using a very aggressive strain of *S. trifoliorum* in controlled environmental screening of red clover plants – resulted in a good correlation with red clover plant survival in the field. Results from Paper I, the controlled environment tests in Paper II and Study B revealed some interesting relationships between different degrees of hardiness and cold-induced resistance to clover rot. A study of differences in gene-expression and accumulated metabolites in red clover plants, before and after hardening and/or *S. trifoliorum* inoculation could lead to the identification of specific genetic or metabolic markers. These would be very useful in future breeding programmes and could provide tools for quicker and efficient selection to improve red clover persistence. Such an investigation has not yet been undertaken for red clover, but one is planned.

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