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Prevalence, species composition, genetic variation and pathogenicity of clover rot (*Sclerotinia trifoliorum*) and *Fusarium* spp. in red clover in Finland

T. Yli-Mattila · G. Kalko · A. Hannukkala · S. Paavanen-Huhtala · K. Hakala

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Abstract The species composition of a total of 173 red clover root fungal isolates from red clover roots from two established organic fields, a field in a transitional phase to organic and from two conventional fields was investigated based on morphology and molecular methods. *Fusarium avenaceum* was the most common *Fusarium* species overall but it occurrred less frequently in older organic fields. *Gliocladium* spp., *Trichoderma* spp. and *Rhizoctonia* spp. isolates were more common in the established organic clover fields, which had been under organic management for more than ten years and in one conventional field, than in a field still in the transitional phase. The taxonomical status of certain *Fusarium*, *Alternaria* and *Sclerotinia* isolates difficult to identify by morphological traits alone could be confirmed by species-specific primers and by comparing their ITS (internal transcribed spacer region) sequences to known sequences. The fingerprinting patterns of RAPD-PCR products could be used for the identification of fungal isolates and for studying the genetic variation among the isolates. Only one of the *Fusarium* isolates originating from apparently healthy red clover roots was clearly pathogenic to germinated red clover seedlings. In detached leaf experiments, the cvs Jokioinen and Ilte were more susceptible to one of the *Sclerotinia trifoliorum* isolates than cvs Betty and Bjursele, while all of them were equally susceptible to two other *S. trifoliorum* isolates. In further greenhouse experiments with intact plants it was possible to slow down the development of clover rot to some extent by means of one of the biological agents tested (*Bacillus subtilis* 10-VIZR, commercial name Alirin B), and almost completely by chemical control.

Keywords Biological control · ITS sequences · Organic farming · RAPD-PCR · Resistance · Root rot

T. Yli-Mattila (✉) · S. Paavanen-Huhtala
Laboratory of Plant Physiology and Molecular Biology,
University of Turku,
FIN-20014 Turku, Finland
e-mail: tymat@utu.fi

G. Kalko
Laboratory of Microbiological Plant Protection,
All-Russia Research Institute for Plant Protection,
Shosse Podbelskogo, 3,
St.-Petersburg 196608, Russia

A. Hannukkala
Plant Production Research/Plant Protection,
MTT Agrifood Research Finland,
FIN-31600 Jokioinen, Finland

K. Hakala
Plant Production Research,
MTT Agrifood Research Finland,
FIN-31600 Jokioinen, Finland

Introduction

Red clover (*Trifolium pratense*) is the main perennial leguminous fodder crop with symbiotic *Rhizobium* bacteria for nitrogen fixation in Finland. Practically

all crop rotations in organic agriculture include red clover, as good yields of cereals can only be obtained when they are grown after an efficient nitrogen fixer. The soil structure is also improved by the large root biomass of red clover. Red clover is often also included in crop rotations in conventional farming because of these benefits. Besides the beneficial effects on soil structure and fertility, red clover is an important forage plant for cattle (Bertilsson and Murphy 2003).

Organic agriculture has become increasingly important in Finland during the last 10–20 years. Organic farming produces only 2% of Finland's total grain yield, but as much as 11% of pea, 10% of rye and 3–4% of hay and silage yield. In contrast, organic milk production in 2007 constituted only approximately 1% (27×10^6l) of total milk production in Finland (www.matilda.fi). One of the main problems in organic agriculture is the low persistence of red clover in the ley. The main reason for this is the poor overwintering and thus non-perenniality of red clover, caused mainly by frost and different plant diseases. The causal agent of clover rot, *Sclerotinia trifoliorum* (also known as *Sclerotinia* crown and stem rot), is economically one of the most important pathogens of young red clover stands in Europe and North America (Ylimäki 1969a; Willetts and Wong 1980; Saloniemi 1993; Öhberg 2008). Clover rot (Fig. 1) may completely destroy large patches in red clover fields during winter. Root rots, mainly caused by *Fusarium* species cause severe injuries in older red clover crops.

In Finland, the severity of clover rot declined in the 1980's compared to the 1950's and 1960's (Ylimäki 1969a; Huokuna 1985), probably due to the decrease in red clover cultivation following the increased use of nitrogen fertilisers. In organic farms where leguminous crops are grown very frequently in the crop sequence, however, there is the risk that the importance of clover rot will again increase. In 1960's PCNB (pentachloronitrobenzene, quintozene) products were successfully applied in clover rot control (Ylimäki 1969a, b). These environmentally questionable compounds have been banned in conventional production for decades and chemical control is out of question in organic agriculture. No biocontrol agents are currently registered for clover rot control in Finland. The management of clover rot is completely based on cultural practices and cultivar resistance. Pure stands of red clover are extremely vulnerable to clover rot attacks and therefore only mixtures of red clover and gramineous species are recommended in production (Huokuna 1985). Some progress in improving genetic resistance especially in late-flowering cultivars has been recently achieved (Öhberg 2008).

Sclerotia of *S. trifoliorum* can survive in the soil for several years (Ylimäki 1969a; Öhberg 2008), but under field conditions they are attacked and degraded by a number of mycoparasites that can penetrate the rind (Bolton et al. 2006). During the summer, *S. trifoliorum* is dormant in sclerotia. In autumn the sclerotia germinate and produce apothecia. High humidity promotes further infection between late autumn and early spring, resulting in the systemic growth of mycelium in the infected plant and others around it.

In the closely related *S. sclerotiorum* the sclerotia germinate and produce apothecia during the summer. *Sclerotinia sclerotiorum* can penetrate the cuticle of the host plant using enzymes or appressoria and through stomata (Bolton et al. 2006). According to Guimaraes and Stotz (2004) oxalic acid produced by *S. sclerotiorum* during infection may open the stomata for the

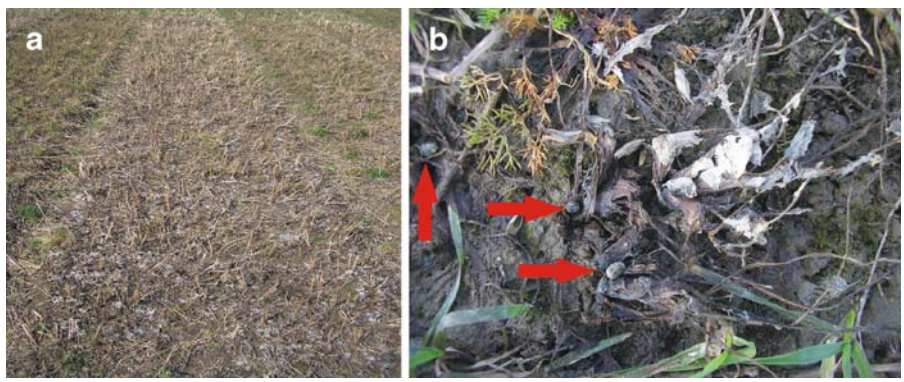

Fig. 1 **a**. Field plot, completely destroyed by *Sclerotinia trifoliorum* in cultivar trials in eastern Finland (Sotkamo), spring 2004. **b**. Damage and sclerotia (shown by red arrows) caused by *Sclerotinia trifoliorum* in the same field plot as in Fig. 1a (photos by Jukka Kemppainen)

invading hyphae. No detailed information on these processes is available for *S. trifoliorum.*

Many *Fusarium* species, along with other soil-borne plant pathogenic fungi, can cause root rot in red clover under certain environmental conditions, and may gradually reduce the number of living red clover plants in the field (Ylimäki 1967; Rufelt 1986; Lager 2002). Most of these fungi are minor pathogens, but in the presence of disease-enhancing abiotic factors or plant stress, caused for instance by frequent cutting or harvesting, the disease may be severe (Rufelt 1986; Lager 2002). While clover rot usually causes damage during the winter in regions with a long lasting and thick snow-cover, root rot causes injuries throughout the growing season (Ylimäki 1967; 1969a). The damping-off form of root rot may cause severe damage to seedlings, while root rot of older plants causes a slow decline of clover in the field.

In the early stages, symptoms of root rot infection may be limited to the cortical region, but later the internal vascular cylinder is damaged and finally destroyed. As a result, the growth and metabolism of the plant are disturbed and the plant slowly declines. Root rot occurs at all times of the year, but the damage is usually greatest in the spring soon after snow-melt, when the clover plants are weakened by the winter (Ylimäki 1967).

Since the morphological identification of fungal species and strains is often difficult, new molecular methods, based on DNA sequences, have been developed. The advantages of these new methods are their rapidity, sensitivity and cost-effectiveness (Janse 1995). RAPD (random amplified polymorphic DNA)-PCR is one of the fingerprinting methods, which can be used for screening differences and similarities between fungal isolates without previous knowledge about their DNA sequences. When species-specific markers are found, the PCR products can be sequenced and more specific SCAR (sequence characterised amplified region) primers can be designed (e.g. Paavanen-Huhtala et al. 2000).

This research was part of an extensive organic farming research programme (2003–2005) gathering together all knowledge of Finnish organic farming and aimed at promoting organic farming in Finland. The programme was financed by the Ministry of Agriculture and Forestry, together with MTT Agrifood Research Finland. The present study is a part of a subproject Red clover efficiently into organically produced milk (www.mtt.fi/research/projects). This project aimed at improving the profitability of organic milk production by increasing the efficiency of red clover cultivation and milk production. The pathogen studies reported here were part of the study to determine ideal variety types and clover-grass mixtures for best winter survival and persistence of red clover in leys.

The main purpose of this paper was to determine the major species associated with organic and conventional red clover, their genetic variation and pathogenicity to red clover. For this purpose, fungal isolates were recovered from root samples of organic, transitional and conventional red clover fields and identified using different morphological and molecular techniques, and pathogenicity tests were performed. The DNA sequences were also used for studying the relationships between *S. trifoliorum* isolates compared to known *Sclerotinia* DNA sequences. In addition, differences in *S. trifoliorum* resistance among four red clover cultivars and pathogenicity among three *S. trifoliorum* isolates were studied in detached leaf experiments. Finally, the inhibitory effects of various fungal and bacterial biological control agents on *S. trifoliorum* infection by one isolate were studied on the red clover cv. Bjursele in greenhouse experiments.

Materials and methods

Fungal isolates and their morphological identification

Symptomless red clover plants were collected from a mixed red clover-grass field in a transitional phase from conventional to organic (sown in 2003) at Jokioinen (60°48′ N, 23°29′ E) (south-western Finland, 7 in plants 2003 and 12 plants in 2004), from two conventional fields (VT and JP) at Marttila (60°63′ N, 23°00′ E) (south-western Finland, 13 plants in 2003 and 16 plants in 2004) used for red clover seed production of cv. Betty (sown in 2002) (Table 1), and from two mixed red-clover-grass organic fields (sown in 2001 and 2002) at Juva (61°53′ N, 27°51′ E) (south-eastern Finland, 8 plants in 2003 and 12 plants in 2004) in September 2003 and July 2004 (Table 2). The fields at Juva have been under organic management since 1986. From a third mixed red-clover-grass research field at Sotkamo (647′ N, 28°23′ E) (transi-

Table 1 Number of fungal isolates obtained from red clover roots in 2003, 2004 and 2006 from a field in a transitional phase from conventional to organic (Jokioinen, sown in 2003) and from conventionally managed fields (Marttila I and Marttila II, sown in 2002)

Year, origin and yield year	Number of plants	Number of isolates	*F. avenaceum* isolates (JIA primers)	*F. avenaceum*/ *F. acuminatum*/ *F. tricinctum*	Other *Fusarium* isolates	*Alternaria* isolates (morphological identication./ *A. alternata*-specific primers)	*Gliocladium*/ *Trichoderma* isolates	*Rhizoctonia* isolates	*Cylindrocarpon*	Other fungi
2003										
Jokioinen (sowing year)	7	11	4	4	1	3/1	0	0	1	2
Marttila (I, 1. yield year)	6	13	6	9	1	0	1	0	0	2
Marttila (II, 1. yield year)	7	15	4	6	3	1/0	0	0	1	4
2004										
Jokioinen (1. yield year)	12	15	3	5	5	0	1	0	4	
Marttila (I, 2. yield year)	8	18	4	5	4	0	3	2	2	2
Marttila (II, 2. yield year)	8	13	5	5	5	0	0	0	0	3
2006										
Jokioinen (3. yield year)	8	17	3	3	5	0	0	0	0	12
Total	56	105	29	37	24	4/1	5	2	8	25

Table 2 Number of fungal isolates obtained from red clover roots in 2003, 2004 and 2006 from a first-year (2003–2004, sown in 2002–2003), second year (2003–2004, sown in 2001–2002) and third year (2006, sown in 2003) organic field from Juva

Year, origin and yield year	Number of plants	Number of isolates	*F. avenaceum* Isolates (JIA primers)	*F. avenaceum*/ *F. acuminatum*/ *F. tricinctum*	Other *Fusarium* isolates	*Alternaria* isolates (morphological identification)	*Gliocladium*/ *Trichoderma* isolates	*Rhizoctonia* isolates	*Cylindrocarpon*	Other fungi
2003										
Juva (1. yield year)	4	20	3	9	3	0	0	2	0	6
Juva (2. yield year)	4	11	2	3	2	0	5	1	0	0
2004										
Juva (1. yield year)	6	11	1	1	2	0	0	2	1	5
Juva (2. yield year)	6	12	1	1	1	0	1	1	2	6
2006										
Juva (3. yield year)	10	14	0	0	3	0	3	0	0	8
Total	30	68	7	14	11	0	9	6	3	25

tional field) in northeastern Finland only sclerotia from dead plants were collected in 2004. Additional sclerotia were obtained from a conventional field at Rovaniemi in northern Finland in 2003. In addition, dead or diseased red clover plants grown in the same fields at Jokioinen (8 plants, Table 1) and Juva (10 plants, Table 2) were collected in May 2006.

The red clover roots were kept in a cold room in plastic bags for up to two weeks before fungal isolation. Fungal isolates were obtained from pieces of surface-sterilised (1% hypoclorite 5 min, 70% ethanol 1 min, washed with sterile distilled water (SDW) and dried with sterile filter paper) red clover roots. Three pieces per root sample were placed on PDA medium (Fig. 2). A total of 70 and 74 fungal isolates were obtained from the apparently healthy root samples of the years 2003 and 2004, respectively. In addition, 31 fungal isolates were collected from the diseased red clover roots in 2006 (Tables 1 and 2). The isolates grown on PDA medium (Fig. 2) were identified by growth and microscopy of morphological traits of spores, conidiophores and spore formation (Barnett and Hunter 1998). The taxonomy and identification keys by Gerlach and Nierenberg (1982) were used for identification of *Fusarium* species.

Most *S. trifoliorum* isolates were obtained from surface-sterilised sclerotia, (70% ethanol ca. 10 s, washed with SDW and dried with sterile filter). The sclerotia were than placed on PDA medium. (Fig. 2). The sclerotia originated from field experiments conducted by MTT with red clover in Lapland (isolates L-110 and L-119), north-eastern Finland (isolates K-1-K-4, Fig. 1) and south-eastern Finland (J-1 and J-2), which were destroyed by *S. trifoliorum* (Table 3). In 2006, *S. trifoliorum* isolates were also obtained from diseased plant roots at Juva and Jokioinen.

Molecular methods

DNA extraction and analyses of pure cultures were performed as described by Yli-Mattila et al. (2004). ITS (internal transcribed spacer) sequences of several *S. trifoliorum* and 28 fungal isolates from red clover roots were compared to known sequences in GenBank to confirm the morphological identification. ITS amplification was also used to check the quality of DNA for PCR. *Fusarium avenaceum* isolates were identified by species-specific JIA primers as described by Yli-Mattila et al. (2004), while *Alternaria alternata* isolates were identified by AA primers as described by Gannibal and Yli-Mattila (2005).

RAPD-PCR analyses were performed as described by Paavanen-Huhtala et al. (2000). Eighteen *Fusarium* isolates from 2003, including eight isolates from Marttila, three from Jokioinen and seven from Juva, were studied with primer 91299. Fourteen of them were used for artificial inoculation. In 2004, several *F. avenaceum* and *Fusarium* sp. strains were analysed with primers 91299 and Y. *S. trifoliorum* strains from Juva, Sotkamo and Lapland were analysed with RAPD-PCR primers OPA3, OPA8, OPA20 (Operon Technologies, Alameda, Ca, USA) and 91299.

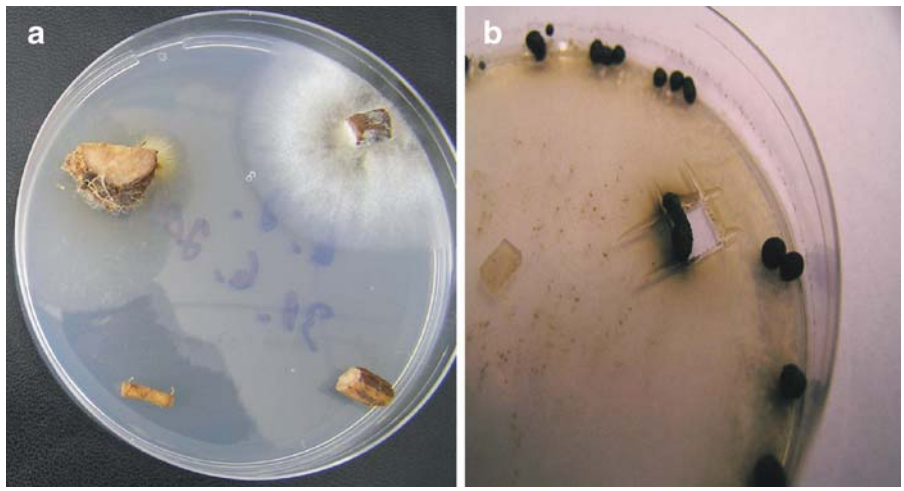

Fig. 2 **a**. Mycelia (*Mucor* sp. left and *Trichoderma* sp. right) grown from surface-sterilised pieces of red clover roots during four days on PDA. **b**. New sclerotia of *S. trifoliorum* were formed on the mycelium, when it reached the edge of the Petri dish or where the mycelium was damaged by cutting

Table 3 *Sclerotinia trifoliorum* strains used in the work

Strains	Geographical origin (latitude and longitude)	Year of isolation
L-119, L-110	Rovaniemi (6630′N, 25°44′ E)	2003
J-2, J-1	Juva (61°53′ N, 27°51′ E)	2004
J-6	Juva (61°53′ N, 27°51′ E)	2006
K-1, K2, K3, K4	Sotkamo (64°7′ N, 28°23′ E)	2004
Jo-14	Jokioinen (60°48′ N, 23°29′ E)	2006

Artificial inoculation with *Fusarium* conidia

The pathogenicity of *Fusarium* isolates from symptomless plants was determined in laboratory tests on inoculated seeds and eventually on the seedlings of cvs Jokioinen and Bjursele. Seven isolates (four *F. avenaceum* isolates, two *F. oxysporum* isolates and one *F. culmorum* isolate) were studied on both cultivars and seven additional isolates were studied on cv. Jokioinen only.

Seeds were surfaced-sterilised with 0.1% $AgNO_3$ for 1 min, washed with SDW and inoculated with a conidial suspension of $0.3–1.5\times10^6$ conidia ml^{-1}. Inocula were prepared by washing the spores from the surfaces of 2–3 week old *Fusarium* cultures growing in Petri dishes on a nutrient-rich medium (potato dextrose agar, PDA) or on a low-nutrient medium (Synthetischer Nährstoffarmer Agar, SNA). The densities of conidia were estimated with a haemocytometer. The seeds were inoculated by soaking in a conidial suspension for 10 min and then lightly dried on sterile filter paper or sterile absorbent cotton and placed in sterile Petri dishes onto moistened filter paper. The experiments were performed in 4 replicates, with 50 seeds per (Petri dish) replicate.

The seeds were incubated in the Petri dishes under constant light (80–90 μmol photons m^{-2} s^{-1}) at 24°C. After 2 days of incubation, the germination of the seeds was observed, and after 2 weeks the severity of disease symptoms on the seedlings was evaluated using a scale with classes: 0 = healthy root, 1 = <10% of the root surface affected by brown lesions, 2 = 10–25% of the root surface covered by brown lesions, 3 = 25–50% of the root surface covered by brown lesions, 4=>50% of the root surface covered by brown lesions or plant dead.

Plant material

The field experiments at Jokioinen, Juva and Sotkamo, from which the fungal samples were collected were part of a larger research project of MTT Agrifood Research Finland dealing with organic red clover.

Four different cultivars of red clover (*Trifolium pratense*) were compared in the study. Jokioinen is a diploid cultivar of Finnish origin (Boreal), which is recommended for southern Finland. Bjursele is a Swedish (Svalöf Weibull) diploid cultivar, which is rather winter-hardy and recommended for the whole of Finland. Betty and Ilte are tetraploids of Swedish (Svalöf Weibull) and Estonian (Jõgeva Plant Breeding Institute) origin, respectively. Betty originates from Bjursele; it is even more winter-hardy, and is recommended for the whole of Finland. Ilte, though less winter-hardy than Betty, has a capacity for very high yields, and is recommended for southern Finland (Kangas et al. 2005). For the leaf tests with all four clover cultivars, plants were grown in the greenhouse in standard potting mixture, and the leaves of two month-old plants were used for tests. The commercial standard potting mixture (Kekkilä, Finland) was based on peat, mixed with 20% of the total volume of mineral soil (33% coarse sand, 33% fine sand and 33% sandy loam). The mixture was fertilised with 1.2 kg m^{-3} of N-P-K fertiliser (6–12–24) and with 6 kg m^{-3} of Mg containing (5%) ground limestone. The pH of the mixture was 6.2.

For the whole-plant tests, Bjursele clover plants were sown on a standard potting mixture and first grown in a growth room with a 12/12 h day (40–55 μmol photons $m^{-2}s^{-1}$)/night regime at about 20–22ºC. When the plants were three weeks-old, they were potted in 8×8 cm pots and transferred to two growth chambers with a 12/12 h day/night regime (12 h light of 190–270 μmol photons $m^{-2}s^{-1}$, 20°C / 12 h in darkness, 10°C. After two weeks growth the plants were transferred to a sterilised growth room with a 12/12 h day (40–55 μmol photons $m^{-2}s^{-1}$)/night regime at 15°C, where they were treated with biological agents within two days of the transfer.

Preparation of *S. trifoliorum* inoculum

The mycelial inoculum was prepared according to the modified method of Rhodes et al. (1989) and Marum et al. (1994). Three mycelial balls with a diameter of about 2–3 cm (grown from three mycelial plugs in one flask during 7 days at 15°C on a rotary shaker in 250 ml of PD broth) were added to 250 ml of water and blended in a Waring Blender for about 5–10 s. The mycelium for leaf inoculum was separated from the water by centrifugation and then blended in the same Waring Blender for 2 min in 200 ml of SDW. Three drops of Tween 80 were added to every 100 ml of inoculum.

Inoculation of detached clover leaves with *Sclerotinia trifoliorum* isolates

Detached leaves of cvs Bjursele, Jokioinen, Betty and Ilte were used for evaluation of the pathogenicity of three isolates, L-119, K-1 and J-2, of *S. trifoliorum*. The clover leaves (about two months-old) were placed in Petri dishes on the surface of 0.5% water agar with 50 mgl^{-1} benzimidazole (modified from Delclos et al. 1997, who used ascospore inoculum). The experiments were performed in 4 replicates, with 6–12 leaflets per (Petri dish) replicate.

The clover leaflets were inoculated with 10 μl drops of inoculum (optical density of 1.3–1.7 at wavelength 750 nm), which were placed in the middle of each leaflet (Efimova 1985). The controls were moistened with 10 μl drops of water (with added Tween 80). The Petri dishes with the inoculated clover leaves were then incubated at 15°C in 12 h day-length (light intensity 40–55 μmol photons $m^{-2}s^{-1}$) for two weeks. Disease symptoms were evaluated using a scale with classes: 0 = healthy leaf, 1 = slight necrosis, 2 = moderate necrosis, 3 = severe necrosis, 4 = leaf dead.

Evaluation of antagonistic activity on intact plants

The effect of commercially available antagonists on *S. trifoliorum* on potted intact five week-old clover plants was studied in an experiment with inoculation of the cv. Bjursele by the aggressive (based on leaf experiments) *S. trifoliorum* isolate L-119.

Biological agents and one fungicide (Rovral, Bayer, active agent iprodion, sold by Kemira GrowHow in Finland) were sprayed one day before *S. trifoliorum* inoculation as follows: Mycostop (*Streptomyces griseoviridis,* Verdera Ltd, Finland) and Alirin B (*Bacillus subtilis* 10-VIZR, Russia, Novikova et al. 2003), 0.6 ml/plant of 0.5% solution; Prestop and Gliomix (*Gliocladium catenulatum,* Verdera Ltd, Finland), 0.6 ml/plant of 2% solution, 1.2×10^6 cfu/plant; and Rovral, 0.6 ml/plant of a solution of 165 mg/100 ml as a chemical control. All the preparates were mixed with deionised water 1–1.5 h before use. The spore amount was 1.5×10^8/plant in Alirin B and 1.5×10^6/plant in Mycostop. The inoculated controls were treated with deionised water (also 0.6 ml/plant). The experiments were performed in three replicates, with five plants per replicate placed on a tray.

The day after spraying with biological agents and fungicide, the clover plants were sprayed with mycelial inoculum of isolate L-119. The optical density of mycelial fragment suspension was 1.05 (750 nm). The amount sprayed was ca. 1 ml per plant. Control plants (water control) were sprayed with water mixed with Tween 80 (3 drops per 100 ml of water). After inoculation, a plastic top was placed over the replicate trays to keep the plants at 100% relative humidity, and the resulting chambers (five plants per chamber) were incubated at 15°C in 12 h day-length (light intensity 40–55 μmol photons $m^{-2}s^{-1}$) for four weeks. The effect of the inoculation (inoculated control) was compared to the control plants sprayed with water (water control). Disease development was rated two and four weeks after the use of biological agents by using the same classes as for clover leaves.

The biological effectiveness (BE_{DSI}) of biological and chemical agents was calculated by the formula:

$$BE_{DSI} = (DSI_c - DSI)/DSI_c$$

DSI disease severity index in samples treated by biological agent

DSI_c disease severity index in inoculated control without biological control.

The disease severity was simply taken as the mean of the five classes assessed. The classes were not linear but more like exponential scaling with 0, 0–10, 10–25, 25–50 and 50–100 %. Thus, the classes 0–4 were converted in the mean percentage values (0, 5, 17.5, 37.5 and 75) before calculating the mean and comparing the biological effectiveness in Table 7.

Statistical analysis

The effect of the *S. trifoliorum* strain on the frequency of severely injuried leaves in different red clover cultivars was analysed using logistic regression analysis as described by Lehtinen et al. (2007). To achieve binomial distribution the original disease ratings 0–2 were reclassified as 0, healthy leaves and mild symptoms, and ratings 3–4 as 1, severe symptoms. In logistic regression the response variable is the probability that an event will occur (e.g. prevalence of severe *Sclerotinia* symptoms), hence the response variable is constrained between 0 and 1. The 'odds' of an event are defined as the probability of the outcome event occurring divided by the probability of the event not occurring. The 'odds ratio' is one set of odds divided by another. An odds ratio of 1 indicates that the event under study is equally likely in both values of the predictor. An odds ratio >1 indicates that the event is more likely in the first value, whilst an odds ratio <1 indicates that the event is less likely in the first value. The data were analysed in three steps. First, odds ratios were calculated for the whole data. The untreated control differed so much from all the isolates that comparisons between different isolates could not be shown. Therefore, in the second step the untreated control was omitted from the analysis and other isolates were compared to isolate J-2 and other cultivars were compared to cvs Betty and Bjursele. In the third step, differences among isolates were studied separately for each of the four cultivars.

Results

Fungal species composition in red clover roots based on morphological characteristics, species-specific primers and ITS sequence analyses

No diseased plants were found in September 2003 or July 2004 at Jokioinen or Marttila. In contrast, at Juva clover rot caused some damage, and at Sotkamo most of the red clover was destroyed in the field plots during the first winter, 2003–2004, by clover rot (Tables 1 and 2). Using species-specific primers it was possible to confirm the morphological identification of 33 *Fusarium* and one *Alternaria* isolates (Tables 2 and 4). One *Rhizoctonia solani* isolate was also found to be mixed with *F. avenaceum* based on species-specific primers. Nineteen of the 70 isolates of the year 2003 and fourteen of the 69 isolates of the year 2004 gave a positive result with JIA primers and could thus be confirmed as *Fusarium avenaceum*, which was the most common *Fusarium* species in all fields studied. Nine isolates of the year 2003 and three

Table 4 Examples of *Fusarium* and other fungal isolates collected from red clover roots from different fields and re-identified by *F. avenaceum/F.arthrosporioides*-specific JIA primer and *A. alternata*-specific AA primer pairs and by comparing their ITS (internal transcribed spacer region) sequences to known ITS sequences

Morphological identification	Code	Molecular identification		
		JIA	AA	ITS
Cylindrocarpon sp.	A1/2	–	n.a.	*Cylindrocarpon destructans/Neonectria radicicola*
Fusarium avenaceum	A4/2	+	n.a.	n.a.
F. avenaceum	3*	+	n.a.	*F. avenaceum/F.tricinctum*
F. avenaceum	VT5/1	–	n.a.	n.a.
Alternaria sp.	A4/1	–	+	n.a.
Alternaria sp.	A3/1	–	–	n.a.
F. avenaceum	VT4/3	–	n.a.	*F. avenaceum/F.tricinctum*
F. avenaceum	5/2	–	n.a.	*F. avenaceum/F.tricinctum*
Rhizoctonia solani/F. avenaceum	6/2;2*	+	n.a.	n.a.
Mucor sp.	8/2;1	–	n.a.	*Mucor* sp.
F. sambucinum	JP10/1	–	n.a.	*F. culmorum*
F. oxysporum	JP11/2	–	n.a.	*F. oxysporum*

n.a. = not analysed

isolates of the year 2004, morphologically identified as *F. avenaceum*, gave a negative result with JIA primers. *Fusarium culmorum* was only found in the conventional fields, while *R. solani*, *Gliocladium* spp. and *Trichoderma* spp. were most common in the organic fields at Juva; *Cylindrocarpon* spp. were more common in 2004 than in 2003 (Tables 1 and 2). There were no clear differences in the number of species in seemingly healthy red clover roots between different fields in 2003–2004.

In 2006, *Fusarium* fungi were found in six of the diseased red clover plants at Jokioinen and three *Fusarium* isolates could be identified as *F. avenaceum* with JIA primers. *S. trifoliorum* was found in one red clover plant at Jokioinen and one at Juva (Table 3). *Fusarium, Mucor*, *Gliocladium*, *Trichoderma* and *Phoma* fungi and unidentified oomycetes were also found at Juva in 2006 (Tables 1 and 2). It was interesting that the *S. trifoliorum* isolate at Juva was isolated together with a *Gliocladium* isolate from a diseased red clover plant, which was growing close to several dead red clover plants. Isolates A4/2 and A4/3 were *F. avenaceum*, while isolate 6/2;2 was a mixture of two species (Table 4). The morphological identification of isolate 5/2 could not be confirmed by species-specific primers, but according to the ITS sequence it was either *F. avenaceum* or closely related *F. tricinctum*.

ITS sequences were not sensitive enough to identify all *Fusarium* isolates at species level, but it was possible to confirm the identification of *S. trifoliorum* isolates K-1, J-2 and L-119 by comparing the ITS sequences (Accession numbers DQ904361 and DQ904362) of the present work to those found previously (Fig. 3). Isolates J-2 and L-119 had one nucleotide difference in the ITS1 region compared to known ITS sequences of *S. trifoliorum*, while in isolate K-1 it was not possible to identify this nucleotide.

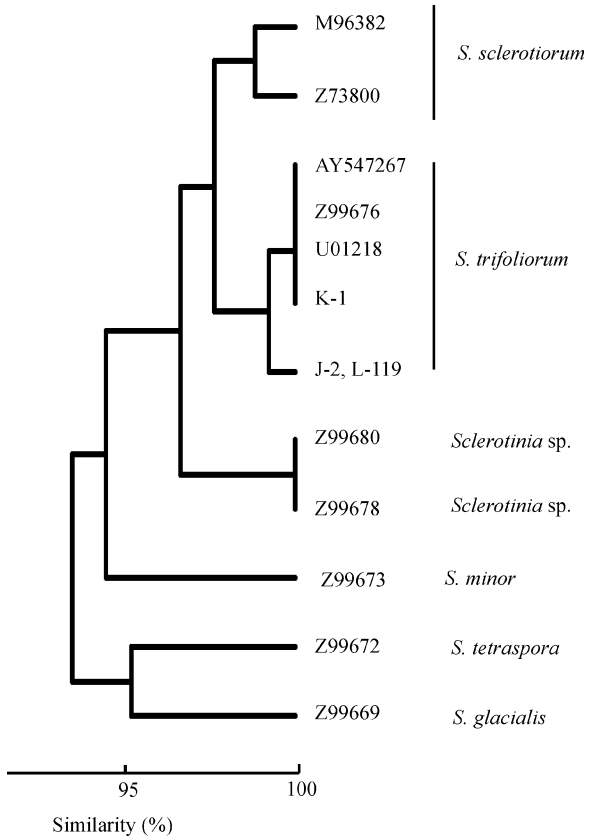

Fig. 3 UPGMA tree based on ITS sequences. Finnish *S. trifoliorum* isolates K-1, J-2, L-119, are grouped together with known *S. trifoliorum* strains.

RAPD-PCR analyses

In 2003 the total amount of polymorphic RAPD-PCR bands in 18 *Fusarium* isolates was 24 (Fig. 4). The number of pair-wise differences between them was 0–11 bands and the distribution of the frequencies of pair-wise differences was normal among all *Fusarium* isolates and within the populations of Juva and Marttila (results not shown). All *Fusarium* isolates of the year 2004 could be distinguished from each other based on the specific RAPD-PCR fingerprinting patterns (Fig. 5), while two *F. avenaceum* isolates of the year 2003 (7;1 and 5*, Fig. 4) had identical RAPD-PCR patterns with primer 91299.

The RAPD-PCR patterns of *S. trifoliorum* isolates were almost identical (Fig. 6). Only isolate J-1 from Juva, which was also macroscopically different from other isolates, had clearly different RAPD-PCR patterns. All the *S. trifoliorum* isolates, except for J-1, formed large numbers of sclerotia at the edge of the Petri dish.

The effect of different *Fusarium* strains on red clover seedlings

Fusarium isolates JP9/2 (*F. culmorum*), 3* (*F. avenaceum*), 7;2 (*F. oxysporum*), A4H1/1 (*F. avenaceum*), JP11/2 (*F. oxysporum*), JP13/1 *(F. avenaceum*), VT3/1 (*F. avenaceum)* from seemingly healthy red clover

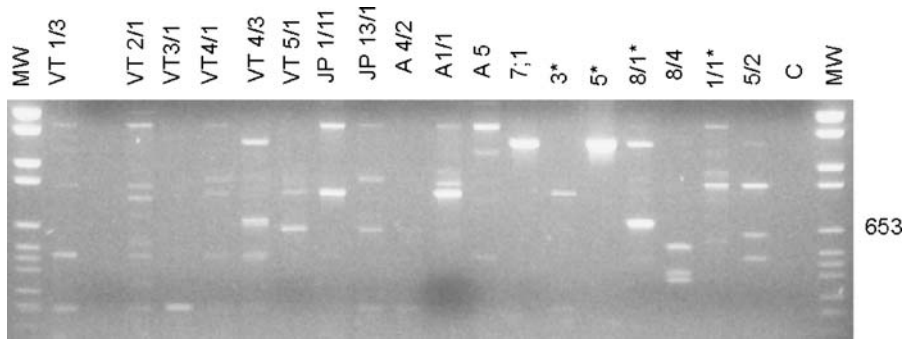

Fig. 4 RAPD patterns with primer 91299 of 14 *F. avenaceum*, and four *Fusarium* sp. (VT 4/3, VT 5/1 and 5/2, VT 4/1) isolates from red clover in 2003 compared to molecular weight marker VI (MW, Boehringer) and negative control (C). VT and JP isolates are from two conventional fields in Marttila, A isolates are from a transitional field at Jokioinen and the rest of the isolates are from an organic field at Juva. Molecular weight markers are 2176, 1766, 1230, 1033, 653, 517, 473, 394, 298, 234, 220 and 154 bp

plants were unable to cause symptoms of root rot or wilt of clover in experiments with the cvs Jokioinen and Bjursele (results not shown). The proportion of ungerminated seeds was higher in Bjursele (25–40%) in comparison to Jokioinen (6–30%).

In further experiments only cv. Jokioinen was tested, because it had a higher germination percentage. Only one of these seven *Fusarium* isolates isolated from seemingly healthy plants was found to be pathogenic to red clover seedlings of the cv. Jokioinen (Table 5). When the identity of the pathogenic *Fusarium* isolate from the infected seedlings was studied by RAPD-PCR fingerprinting, it was confirmed by RAPD-PCR primers 91299 and OPA3 (results not shown) that the isolate from the infected seedling was the same as the isolate 5/2 by which the seedlings were infected.

Response of red clover cultivars to *Sclerotinia trifoliorum* strains in detached leaf experiments

The infection first resulted in a necrotic spot around the inoculum. During the next two weeks the inoculation infected the rest of the leaflets, while in control plants no necrotic spots were formed around the droplets of water (Fig. 7). All cultivars were susceptible to isolates L-119, K-1 and J-2. Cultivar Ilte was equally susceptible to all three isolates tested, while other cultivars were significantly more susceptible to isolates L-119 and K-1 than to isolate J-2 (Table 6).

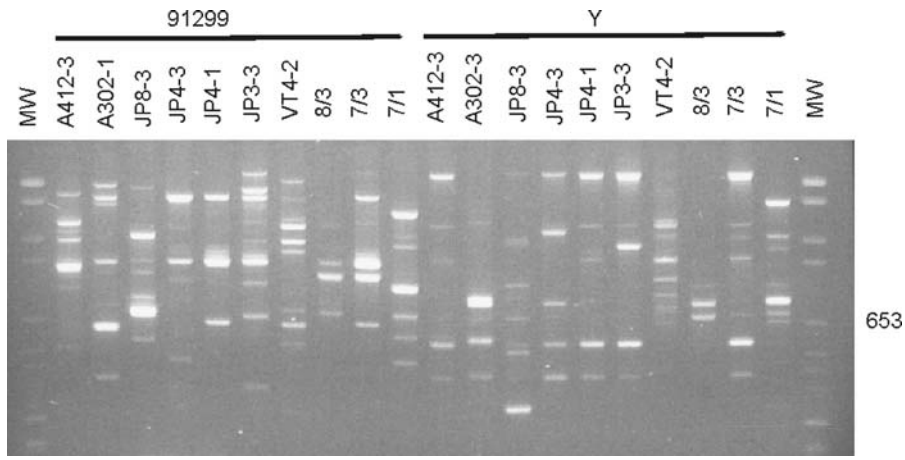

Fig. 5 RAPD patterns with primers 91299 and Y with 5 *F. avenaceum* (A3-02-1, JP4-1, JP3-3, VT4-2 and 7-3) and five *Fusarium* sp. (A412-3, JP8-3, JP4-3, 8-3 and 7-1) isolates from red clover in 2004 compared to molecular weight marker VI (MW, Boehringer) and negative control (C). Isolate markers and molecular weight markers as described in Fig. 4

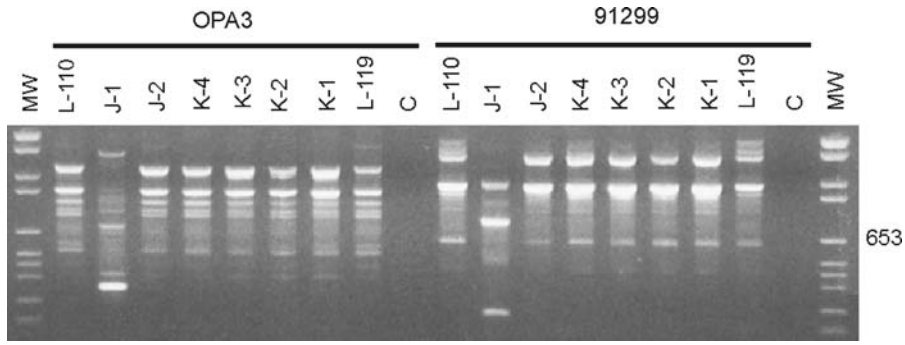

Fig. 6 RAPD patterns of *S. trifoliorum* isolates (L from Lapland, J from Juva and K from Sotkamo) from red clover with primers OPA3 (on the left) and 91299 (on the right) compared to molecular weight marker VI (MW, Boehringer) and negative control (C)

The risk of getting severe symptoms with inoculation by isolate J-2 was ca. 38 and 19 times higher in cv. Ilte and 5 and 3 times higher in cv. Jokioinen in comparison to cvs Bjursele and Betty, respectively, which was statistically highly significant in all cases.

The efficacy of fungicides and biocontrol agents on intact plants

Isolate L-119 was also more aggressive than isolate J-2 in the whole-plant experiments. In the preliminary experiments with J-2 only 60% of the red clover plants of cv. Bjursele developed symptoms, and none was killed two weeks after the treatment (results not shown). With isolate L-119 about 93% of the plants of the cv. Bjursele developed symptoms and 33% were killed within two weeks after inoculation (Table 7). The fungicide Rovral was most effective against *S. trifoliorum* isolate L-119. The most effective biological agent was *B. subtilis* 10-VIZR, Alirin B (All-Russia Institute for Plant Protection), while Prestop (Verdera Ltd.) had only a slight positive effect. The preparates Mycostop and Gliomix (Verdera Ltd.) did not show any effect against *S. trifoliorum* (Table 7). The results obtained from converted DSI values were generally in agreement with those obtained from original DSI values (results not shown).

Discussion

In 2004, *S. trifoliorum* was common only in the northernmost experimental field at Sotkamo. In southern Finland, *S. trifoliorum* was rare in 2003–2006 and it was only found in one apparently healthy

Table 5 Development of disease symptoms of clover seedlings ($\bar{x} \pm s\bar{x}$) under artificial inoculation of seeds of the cv. Jokioinen with *Fusarium* isolates in Petri plates

Fusarium strain	Morphological identification	Molecular identification JIA/ITS	% of ungerminated seeds on 2nd day of incubation	Disease severity index
1/1	*F. a.*	+/n.a.	23.0±8.01	0
5/2	*F. a.*	-/*F. aven./F. tricinctum*	17.5±6.02	2.63±0.76
8/4	*F. a.*	-/?	24.5±14.32	0
A3HO2	*F. a.*	+/*F. aven.*	14.5±7.06	0
JP10/1	*F. s.*	-/*F. culm.*	20.0±12.06	0
JP11/1	*F. a.*	+/*F. aven.*	15.5±9.14	0
JP9/1	*F. s.*	-/n.a.	26.0±13.5	0
Control			20±10.71	0

Molecular identification was based on *F. avenaceum*-specific JIA primers and ITS sequences. n.a. = not analysed. *F. s.* = *F. sambucinum* F. a. = *F. avenaceum* + = positive reaction with *F. avenaceum*-specific primers JIAf/r. - = negative reaction with *F. avenaceum*-specific primers JIAf/r

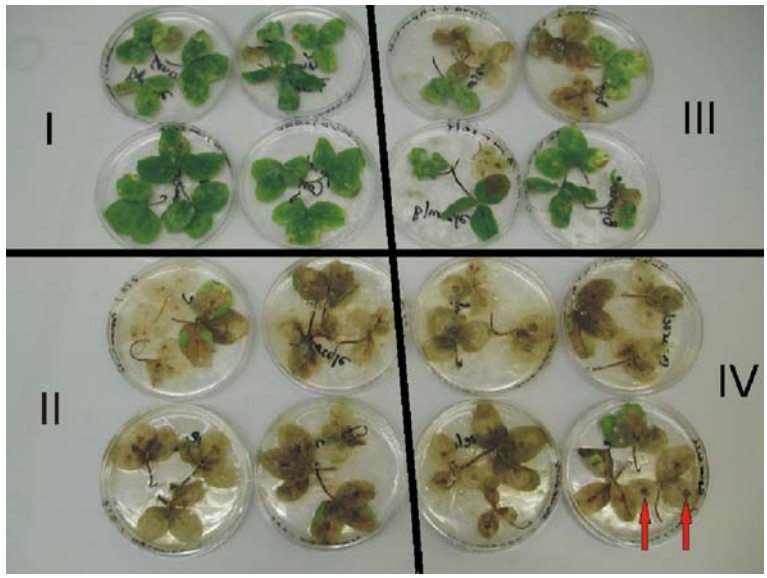

Fig. 7 Bjursele cultivar two weeks after artificial inoculation with three *S. trifoliorum* strains. I = control, II = L-119, III = J-2, IV = K-1. Necrotic spots shown by arrows in two leaflets inoculated with K-1

plant at Juva in 2004 and in two diseased plants at Juva and Jokioinen in 2006. Only cv. Betty survived to some extent to the following year during the first winter after sowing at Sotkamo. The relatively high root weight compared to lower above-ground growth may be an advantage for the survival of red clover during the first winter after sowing, when the damage caused by clover rot usually is most severe (Hakala and Jauhiainen 2007). However, the better resistance of cv. Betty against *S. trifoliorum* shown in the leaf experiments was probably the main reason for its better survival. The higher degree of damage in northern Finland as compared to southern Finland and the better resistance of cv. Betty as compared to

Table 6 Percentage of severe disease symptoms on leaflets of red clover cvs Jokioinen, Ilte, Bjursele, Betty artificially inoculated with different *Sclerotinia trifoliorum* isolates

Clover cultivar	Control	*S. trifolium* isolates			Mean	Odds ratio cultivar vs. Betty	Odds ratio cultivar vs. Bjursele	Odds ratio by J-2 in cultivar vs. Bjursele/ Betty
		J-2	K-1	L-119				
		% of severely injured leaflets (*n*)						
Betty	0 (41)	67 (39)	92 (39)	97 (36)	65	–	1.22	2.00/–
Bjursele	3 (36)	50 (37)	100 (36)	100 (40)	64	0.82	–	–/0.50
Ilte	0 (30)	97 (39)	97 (36)	97 (36)	73	8.06***	9.80***	38.46***/19.00***
Jokioinen	0 (36)	84 (45)	100 (42)	100 (42)	73	3.69***	4.50***	5.43***/2.71*
mean	1	75	97	99				
Odds ratio isolate vs. control (95% Cl)	–	1000***	1000***	1000***				
Odds ratio isolate vs. J-2 (95% Cl)	–	–	14.20***	29.43***				

n = number of leaflets tested. Logistic regression cannot be calculated for isolates K-1 and L119 separately, because too many cells contain 100% injured leaves

*Odds ratio values differ at 5% level. ***Odds ratio values differ at 0.1% level

Table 7 Effectiveness of biological agents in laboratory experiment with plants of cv. Bjursele artificially inoculated with *S. trifoliorum* isolate L-119 two and four weeks after treatment

Treatment	Week	Disease severity (%) after two (above) and four (below) weeks $\bar{x} \pm s\bar{x}$	Biological effectiveness after two and four weeks (%)	Diseased (%) after two and four weeks ($\bar{x} \pm s\bar{x}$)	Biological effectiveness after two and four weeks (%)	Dead (%) after two and four weeks ($\bar{x} \pm s\bar{x}$)	Biological effectiveness after two and four weeks (%)
Water control	2	10.5±4.3	–	66.7±17.6	–	0	–
	4	20.8±7.0	–	80.0±44.7	–	0	–
Inoculated control (L-119)	2	43.5±2.5	–	93.3±6.7	–	33.3±6.7	–
	4	67.5±4.33	–	93.3±6.7	–	86.7±6.7	–
Mycostop + L-119	2	49.3±13.2	0	100	0	46.7±26.7	0
	4	75	0	100	0	100	0
Prestop + L-119	2	38.5±9.0	11.5	100	0	33.3±6.7	0
	4	73.8±1.2	0	100	0	93.3±6.7	0
Alirin B + L-119	2	17.5±3.6	59.8	73.3±17.6	21.4	6.7±6.7	80.0
	4	65.3±5.9	3.2	100	0	73.3±6.7	15.5
Gliomix + L-119	2	53.2±5.6	0	93.3±6.7	0	53.3±17.6	0
	4	71.3±2.2	0	100	0	86.7±6.7	0
Rovral + L-119	2	14.8±7.4	65.9	53.3±29.1	42.9	6.7±6.7	80.0
	4	28.3±7.26)	58.0	100	0	6.7±6.7	92.3

Values are means of results from three trays with five plants.

Bjursele in northern Finland are in agreement with the results obtained in Sweden (Öhberg 2008; Öhberg et al. 2008).

Fusarium avenaceum and *F. tricinctum* were the most common fungi isolated from apparently healthy red clover roots, especially in conventionally managed fields. *Rhizoctonia solani* was more common in organic than in conventionally managed fields. This is in accordance with previous studies (Ylimäki 1967; Lager 2002). Interestingly, also *Gliocladium* and *Trichoderma* species, many of which have been used for biological control, were more common in the organic fields at Juva than in the transitional field at Jokioinen and in the conventionally managed fields at Marttila. *Fusarium culmorum* was only found in a conventionally managed field at Marttila with a long history of cereal plants.

The morphological identification of several fungal isolates, including three *S. trifoliorum* strains, was confirmed by molecular analyses, although *S. trifoliorum* strains J-2 and L-119 had one nucleotide difference from known *S. trifoliorum* strains from North America and Asia. Based on the totally different RAPD-PCR patterns and the lack of sclerotia, it was concluded that isolate J-1 was actually not *S. trifoliorum*. The ITS sequences of *S. trifoliorum* in the present study are the first from European *S. trifoliorum* isolates. According to the ITS sequences *S. trifoliorum* is closely related to *S. sclerotiorum*, which is in accordance with the results of Holst-Jensen et al. (1998) and Carbone and Kohn (2001).

Species-specific primers could be used for confirming the morphological identification of *Fusarium* and *Alternaria* isolates. JIA primers can detect 95% of *F. avenaceum/F. arthrosporioides* isolates from cereal grains identified by morphological characters and it is also possible to identify morphologically-degenerated *F. avenaceum/F. arthrosporioides* isolates by using this primer pair (Yli-Mattila et al. 2004). According to Gannibal and Yli-Mattila (2005) the *A. alternata*-specific primer pair is also specific to the group of species closely related to *A. alternata.* In the present work most of the *F. avenaceum* isolates could only be identified by JIA primers.

This is the first RAPD-PCR study of *S. trifoliorum* isolates. Based on RAPD-PCR analyses the *S. trifoliorum* isolates showed very little variation, while the variation between *F. avenaceum* isolates was high. This is in accordance with earlier studies in the case of *F. avenaceum* in Finland (Yli-Mattila et al. 2004). The lower variation in *S. trifoliorum* in the RAPD-

PCR analysis is in accordance with the low variation obtained in the closely related *S. sclerotiorum* in Canada (Hambleton et al. 2002). On the other hand, based on previous studies in North America (Renhström and Free 1993), half of the ascospores produced by *S. trifoliorum* were heterothallic, which would suggest a higher level of variation. The situation may be different in Finland and the bigger homothallic ascospores may survive better here, at least in the present climatic conditions, which might explain the lack of variation. Despite the genetic similarity between the *S. trifoliorum* isolates, differences were found in their aggressiveness, and a new ITS sequence type was found in Finland. Because of many open questions, more *S. trifoliorum* isolates from different parts of Finland are required to confirm the RAPD-PCR results. The available *S. sclerotiorum* genome sequence (http://www.broad.mit.edu) will facilitate the molecular work with *S. trifololiorum* in the future.

In the present work only one clearly pathogenic isolate against red clover seedlings was found among the 14 *Fusarium* isolates from healthy red clover roots, while in a previous study (Ylimäki 1967) most of the isolates from diseased roots were pathogenic. This difference in results was probably due to the fact that in the present work the isolates of the years 2003–2004 for the pathogenicity studies were obtained from seemingly healthy plants. Thus, root rot was not a problem in any of the studied field plots during the growing seasons 2003 and 2004.

In 2003–2005 clover rot caused damage in northern and eastern Finland, which has a long winter with thick snow cover. In southern and western Finland no damages were reported, although 40–50 years earlier clover rot was common everywhere in Finland. According to the results of the present study the cvs Bjursele and Betty are less susceptible to isolate J-2 from south-eastern Finland than the cvs Jokioinen and Ilte. All of them were susceptible to isolates L-119 from Lapland and K-1 from north-eastern Finland in leaf experiments, although Betty was shown to have some resistance against clover rot in field plots even at Sotkamo. In Sweden too, the most aggressive clover rot isolates have also been collected from the northern part of the country (Öhberg 2008).

In the leaf and whole-plant tests small spots (different from those of *Sclerotinia*) were observed on several leaves of the control plants, but there were no dead plants in the control treatment. The spots in the control plants may be due to abiotic physiological plant disorders connected to the artificial conditions of growing the test plants in chambers with artificial light. Of the four biological agents studied, only Alirin B was able to slow down the development of clover rot and the death of red clover plants. In 2008 it was permitted to use Alirin B in Russia in several plants (http://www.mcx.ru/documents/section/show/4016.89.htm). Sclerotial mycoparasites *Coniothyrium minitans* (Diamantopoulou et al. 2000; Li et al. 2006, Partridge et al. 2006) and *Sporidesmium sclerotivorum* (del Rio et al. 2002) have provided adequate control of the closely related pathogens *S. sclerotiorum* and *S. minor*. In a recent study *C. minitans* from the commercial product Contans®WG was found to have some effect against clover rot sclerotia in naturally infected red clover fields and in laboratory experiments with detached leaflets (Öhberg 2008) Further field and laboratory studies are required to show, how effective different biological control agents are against *S. trifoliorum*.

In organic fields it is necessary to use all possible ways to protect red clover against pathogens: cultivating the least susceptible cultivars, applying optimal crop rotation and growth conditions and the optimal composition of red clover and grass seeds, and using biological control at all stages of the life cycles of the pathogens (e.g. ascospore germination, growth of mycelium in soil, formation of sclerotia) might prove useful as well in the future. Field experiments with naturally and artificially infected plants during the winter time are required to confirm whether the effects of the biological agents, in combination with other methods, are sufficient to protect red clover plants under field conditions. In conventionally managed fields, chemical control agents are very effective, and they could also be used in combination with biological control agents (Diamantopoulou et al. 2000). But currently there is no chemical control agent allowed for chemical control in red clover in Europe.

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