Determining *Leptosphaeria* species on Swedish oilseed rape and monitoring responses to *Fusarium avenaceum and Fusarium graminearum* in timothy populations

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Abstract

Background

Phoma stem canker or black leg disease is one of the most important disease affecting the oil seed rape worldwide. The disease is caused by the two pathogen species *Leptosphaeria maculans* and *L. biglobosa*. In this study genomic information of *L. maculans* and *L. biglobosa* was used to determine their presence in materials collected in the county of Skåne, southern Sweden in 2020. The second part was carried out to understand if timothy is susceptible to pathogens that also cause disease on red clover. *Sclerotinia trifoliorum, Fusarium avenaceum* and *F. graminearum* were used to score their pathogenicity on timothy.

Results

The results showed that *L. maculans* and *L. biglobosa* co-existed in the oilseed rape stem master samples from the five fields analyzed. Pathogenicity observations showed that *F. avenaceum* and *F. graminearum* can cause symptoms similar to crown rot and root rot diseases seen on other crops whereas *S. trifoliorum* did not incite any disease.

Conclusion

This work should be seen as first preliminary data on potential disease problems in Sweden. *Leptosphaeria maculans* and *L. biglobosa* are now established in southern part of the country. These fungal pathogens constitute a potential future threat to *Brassica* crops particularly in light of climate change. *Fusarium avenaceum* and *F. graminearum* cause symptoms on timothy similar to root rot and crown rot on red clover and cereals. If the results could be further validated, they call for new efforts on resistance breeding.

Key words: *Leptosphaeria maculans*, *Leptospharia biglobosa*, *Fusarium avenaceum*, *Fusarium graminearum*, *Sclerotinia trifoliorum*, black leg disease, root rot and crown rot

Popular science summary

Blackleg disease on oilseed rape is caused mainly by two fungal species *Leptosphaeria maculans* and *L. biglobosa*. Disease severity and yield loss can vary with geographic distribution, climatic conditions, cultivars and crop rotation schemes worldwide. Most often these two pathogens co-exist in the crop. This mixed infection can worsen disease severity. Cultural measures such as crop rotation, utilizing of fungicides and tillage are some measures that can be used as disease control. Other than those methods, growing resistant cultivars have become the most promising way of controlling this disease. However the fast-evolving nature of this pathogen is now making it most challenging to use resistant cultivars because the pathogen can overcome the resistance within a few years. Even though the two pathogens were identified to co-exist on oilseed rape grown in southern part of Sweden, blackleg disease is not one of the most serious problems on Swedish oilseed crops. Its severeness could however, increase due to warmer winters and more humid conditions that can occur due to climate change.

Timothy is an important perennial forage grass in many temperate regions. It is considered the most widely grown grass species in Nordic countries to produce high-quality silage and hay. It performs well under a wide range of environmental conditions and most importantly it has winter hardiness. It is commonly used in mixed pastures and can grow compatibly with perennial legumes such as red clover. *Fusarium* species are common soil-borne fungi that attack a broad range of plant species including grass crops. In this study it was found that two *Fusarium* species that attack red clover and cereals also cause disease on timothy. Further experiments and field tests are required to follow up the observations generated in this MSc study.

Table of content

1. Introduction

This work contains two parts, one on oil seed rape (*Brassica napus*), and the other on timothy (*Phleum pratense*) our most common forage grass. Prevalence of different pathogens and disease interactions were monitored and different methods applied to broaden the learning outcomes.

The hemibiotrophic fungus *Leptosphaeria maculans* (anamorph: *Phoma lingam*), causing blackleg or stem canker disease, was described in 1849 as *Phoma lingam*. The sexual stage was first identified 1957 in New Zealand (Williams, 1992). This fungal pathogen is an ascomycete belonging to the class Dothideomycetes (Loculoascomycetes), order Pleosporales. *L. maculans* colonizes crucifers (Brassicaceae) and causes disease mainly on *Brassica* crops (Fitt et al., 2006). Epidemics of blackleg have in the history been severe in Australia and after that the disease was reported in Canada, and Western Europe (West et al., 2001). The disease is now reported from several other continents (Fitt et al., 2006). Disease epidemics in Europe is known to start with airborne ascospores from debris during autumn. Timing of ascospore release may vary between countries and frequencies of harvested oilseed rape fields. Asexual conidia are visual on cotyledons and both asexual and sexual spores can from this stage jointly spread the disease, not least during wet conditions. Infection takes place primarily via stomatal openings, alternatively via wounds (Chen & Howlett, 1996). Besides leaf infection, *L. maculans* can grow systemically in the stem and cause crown canker (Hammond et al., 1985). Seeds have also been found to spread the fungus (Fernando et al., 2016).

The prevalence of a sexual stage promotes genetic variation, and *L. maculans* has been divided into several species-groups over time (Mendes-Pereira et al., 2003). *L. biglobosa* was first described as a weakly-virulent strain of *L. maculans* but is now considered to be a closely related species (Dilmaghani et al., 2009). Phylogeny based on a handful of conserved sequences has further divided *L. biglobosa* into seven subclades: brassicae, canadensis, thalspii, erysimii, australensis,

occiaustralensis and americensis (Zou et al., 2019). Whether this sequence polymorphism observed will lead to real species development remains to be seen.

The genome size of *L. maculans* was estimated to 34 Mb by genetic mapping (Kuhn et al., 2006), a work which was followed by Sanger sequencing of strain JN3 (Rouxel et al., 2011). Genes encoding effectors (secreted proteins or metabolites that promote disease) were located in blocks of AT-rich sequences that occupy approximately a third of the genome of 43.76 Mb. The first *L. biglobosa* sequence analysis revealed a somewhat smaller genome of 31.788 Mb (Grandaubert et al., 2014). By using long-read sequencing technology, two *L. maculans* strains (JN3 and Nz-T4) and the *L. biglobosa* strain (G12-14) has further refined the genome information (Dutreux et al., 2018).

Stem canker is not one of the most serious diseases in Sweden on oilseed rape. Its severeness could however, increase due to warmer winters and more humid conditions allowing sexual spores to form, spores to survive and spread between fields. In this work the aim was to use the genomic information of *L. maculans* and *L. biglobosa* to determine their presence in materials collected in the county of Skåne 2020.

The second sub-part of the study aimed at understanding if timothy (*Phleum pratense*) is susceptible to pathogens that also cause disease on red clover (*Trifolium pratense*), which is a species sown in mixture with timothy to increase protein content in animal feed. Timothy is a winter-hardy, day-length neutral and perennial grass grown in temperate regions throughout the world (Stewart et al., 2011). The tetraploid *P. alpinum* and diploid *P. bertolonii,* two wild species, are thought to be ancestral species to the hexaploid *P. pratense* (Cai & Bullen, 2011). Timothy is an outcrossing species thus cultivars constitute of populations. Soil-borne pathogens are of importance for survival and yield of perennial crops. Knowledge on diseases on timothy is however thin particularly for Swedish conditions. In this study, we were interested to know if pathogens that attack red clover, such as *Sclerotinia trifoliorum* and Fusarium species that attack red clover and cereals, also cause disease on timothy and if there is genotypic variation in provided timothy populations.

2. Materials and methods

2.1. Brassica plant materials, *Leptosphaeria* isolates and their culturing

Ten to fifteen winter oilseed rape (*Brassica napus*) stems were collected at each of five field sites in the county of Skåne, southern Sweden in 2020 and they were dried at room temperature. Two cm^2 pieces of potentially diseased tissue with symptoms were peeled off from each stem to generate 1g in total. The materials were ground into fine powder using a tissue lyzer (Tissue lyzer II Retsch, Germany) and stored at -20° C until DNA extraction. Each master sample was composed of at least three sub-samples of 1 g tissue. Three biological replicates were prepared for DNA analysis of each master sample.

Isolates used in long-read genome sequencing (Dutreux et al., 2018), *L. maculans* (JN3 and Nz -T4) and *L. biglobosa* (G12-14), were used for comparisons. JN3 is a reference sequence isolate of European origin (Rouxel et al., 2011) whereas Nz-T4 originates from New Zealand (Dutreux et al., 2018). *L. biglobosa* isolate G12-14 was isolated in France 2014. The *Leptosphaeria* strains were grown on potato dextrose agar (Sigma-Aldrich, USA) at 21º C in darkness for 14 days. Three mycelium plugs (5 mm diameter) of each isolate were transferred into 100 ml of potato dextrose broth (Becton, Dickinson and Company, USA) and incubated at 21° C in darkness. After 14 days mycelia were harvested, air-dried, ground into fine powder in liquid nitrogen using mortar and pestle and stored at -20° C until DNA extraction.

2.2. DNA extraction

Genomic DNA was extracted from the *Leptosphaeria* reference strains and the stem samples using a cetyl trimethyl ammonium bromide (CTAB) protocol (Möller et al., 1992) with minor modifications. Briefly, 600 µl of 3% CTAB extraction buffer was added to 100 mg ground powder. Samples were incubated at 65° C for 1hr followed by RNaseA treatment. After centrifugation at 13,000 rpm for 10 min, the

supernatant was purified using extraction with phenol:chloroform:isoamyl alcohol (Carl Roth, Germany). DNA was precipitated with one volume of isopropanol. The pellet was washed with 70% ice-cold ethanol, dried and re-suspended in sterile water. The extracted DNA was quantified using NanoDrop ND-1000 spectrophotometer and stored at -20° C until further use.

2.3. PCR analysis

Species-specific primers of *L. maculans* or *L. biglobosa* designed by Liu et al. (2006) were used in PCR analysis. For both species those primers amplified internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. Briefly, a common reverse primer, LmacR (5'-GCAAAATGTGCT GCG CTCCAGG-3') was combined with the forward *L. maculans*-specific primer LmacF (5'-CTTGCCCACCAATTGGATCCCCTA-3') or the *L. biglobosa*-specific primer LbigF (5'- ATCAGGGGATTGGTGTCAGCAGTTGA-3') in the PCR mixture. Expected size of amplification products for *L. maculans* and *L. biglobosa* were 331 bp and 444 bp, respectively. Each reaction of 50 µl contained 1 µl genomic DNA, 10 µl of 5x High-Fidelity buffer, 1 µl of 10 mM dNTP, 0.5 µl Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Lithuania), 1.5 µl of 10 µM forward primer (*L. maculans* or *L. biglobosa*), 2 µl of 10 µM common reverse primer and 34 µl of sterile water. Thermal cycling was performed as using a Bio Rad MyCycler with following conditions: initial denaturation period of 95℃ for 2 min followed by 30 cycles of 95 ℃ for 15 s, 70° C for 30 s, 72° C for 1 min and final extension of 72° C for 10 min. PCR amplified products were visualized on 1% agarose gel stained with Midori green (Nippon Genetics Europe GmbH, Germany).

2.4. Sanger sequencing and analysis

PCR products amplified from DNA of infected stem samples and *Leptosphaeria* reference strains were purified using Gene JET PCR purification Kit (Thermo Fisher Scientific, Lithuania) and sent for Sanger sequencing (Eurofins, Cologne).

The sequences were first edited to remove low quality sequences with SnapGene 5.2 software (https://snapgene.software.informer.com/5.2/). Homology searches were performed with the basic local alignment search tool (BLAST; www.ncbi.nlm.nih.gov), and multiple sequence alignment was carried out using T-COFFEE multiple sequence alignment server (http://tcoffee.crg.cat/apps/tcoffee/do:regular) to determine potential presence of single nucleotide polymorphisms (SNPs). Box Shade server (version 3.1) was used to present aligned results in RTF format (https://embnet.vital-it.ch/software/BOX_form.html). Evolutionary analysis was performed by Maximum likelihood method using MEGA7 version 7.0 (https://www.megasoftware.net/) as explained by Kumar et al. (2016).

2.5. Quantitative PCR (qPCR) analysis

The amounts of *L. maculans* and *L. biglobosa* DNA present in each of the infected stem samples were quantified using a SYBR green qPCR analysis with species-specific primers, LmacF/LmacR and LbigF/LmacR as described above. Each reaction volume was 20 µl, containing 10 µl of Bio Rad SYBR green super mix, 6.3 µl nuclease free water, 0.6 µl (10 µM) of each primer and 2.5 µl (50 ng total) sample DNA. The thermal cycling conditions as explained by Huang et al. (2014) consisted of an initial denaturation for 2 min at 95° C followed by 40 cycles of 30 s at 60° C, 45 s at 72° C and 15 s at 83° C and an additional melting curve with thermal profile conditions of 1 min at 95 °C, 1 min at 60° C and 15 s at 95° C was added to stop the reactions. A standard dilution series of 100000, 10000, 1000, 100 and 10 pg DNA of the three *Leptosphaeria* reference strains was used in each qPCR run to produce standard curves. The slope of the standard curve was used to determine the PCR efficiency (E = 10-1/slope -1). The amount of *L. maculans* or *L. biglobosa* DNA in each sample was estimated by comparing the obtained data to the standard curve. The result was converted into ng *Leptosphaeria* DNA per 100 mg *B. napus* stem biomass dry weight. All calculations were performed as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Bio system, USA) and as explained by Tzelepis et al. (2017) and Martin

et al. (2011). Statistical significance of differences in fungal DNA amounts between samples was calculated using Student's t-test.

2.6. Screening timothy populations against three fungal species

Timothy seedlings were grown in hydroponic medium by following the method developed for *Arabidopsis* (Bindschedler et al., 2008). Briefly, sterilized seeds were transferred to 200 µl pipette tips filled with molten (50-60˚C) half-strength hydroponic solution containing 0.6% agar (Appendix Table 1). The tip boxes were transferred to the growth room for germination under 22 ± 1° C and 16 h light conditions with 120 µE $m^{-2}s^{-1}$ light intensity. After four days of germination, one third of the pipette tips were cut to allow root growth and the tips containing seedlings were transferred into a perforated lid of a black-painted and surface-sterilized polyethylene 21 cm x 15 cm boxes (IKEA, Sweden) filled with 2 l full-strength hydroponic growth solution (Appendix Table 1). Boxes with seedlings were then transferred to the growth room for further growth. Three-weeks old plants were used for infection assay.

Fusarium avenaceum, *F. graminearum* and *Sclerotinia trifoliorum* were used for the infection studies. Their identity was confirmed by ITS sequencing using universal primer pair ITS1-ITS4 (ITS1: 5' TCCGTAGGTGAACCTGCGG 3', ITS4: 5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990).Briefly the amplified products were observed using gel electrophoresis and purified from the gel using Gene JET Gel extraction Kit (Thermo Fisher Scientific, Lithuania). The purified products were sent for sequencing. These three isolates were maintained on potato dextrose agar (Sigma Aldrich Co, USA) at 21º C in darkness. Ten-day-old *Fusarium* fungal cultures grown in Czapek Dox Broth (Duchefa Biochemie, the Netherlands) and ten-day-old *S. trifoliorum* grown in potato dextrose broth (Becton, Dickinson and Company, USA) were used as inoculum. For *Fusarium* fungal isolate, two boxes, each containing 20 plants were inoculated by adding spore suspension (approximately 30,000 spores/ml) to the hydroponic solution whereas for *Sclerotinia* fungal isolate, inoculation was carried out by adding approximately 2 x 10^6cfu /ml to the hydroponic solution. Control plants were similarly treated with

sterile distilled water instead of spore suspension. The infection response was assessed for 14 days and disease scoring (Table 1) was carried out according to Jambagi et al. (in preparation). Plants were scored on a scale from 0 to 6 and the disease severity index (DSI) was calculated and expressed in percentage of diseased plants out of the total plants inoculated. Ten timothy populations (provided by Linda Öhlund at Lantmännen) containing 40 plants for each treatment were included in this analysis. It took five weeks to score one population of timothy.

Table 1: Disease severity assessment criteria

Disease development	**Scale**	**Days post- inoculation**
Healthy/White roots	0	2
Mycelial growth on roots	1	4
Discoloration of roots- brown roots	2	6
Brown to dark brown roots often associated with increasing intensity.	3	8
Dark brown to black roots and yellowing of leaves	4	10
Blackening of roots and browning of leaves often associated with increasing intensity	5	12
Rotten roots/dead plant	6	14

3. Results

3.1. *L. maculans* and *L. biglobosa* are present in Swedish oilseed rape samples

PCR amplification for five samples obtained from Swedish oilseed rape stem samples using LmacF/LmacR and LbigF/LmacR primers showed for each sample DNA bands matching to the size of reference fungal strains, *L. maculans* or *L. biglobosa* (Figure 1). For each sample, three replicates were used to amplify partial sequence of ITS1 and 2 and complete sequence of 5.8rRNA region of *L. maculans* and *L. biglobosa* using species-specific primers.

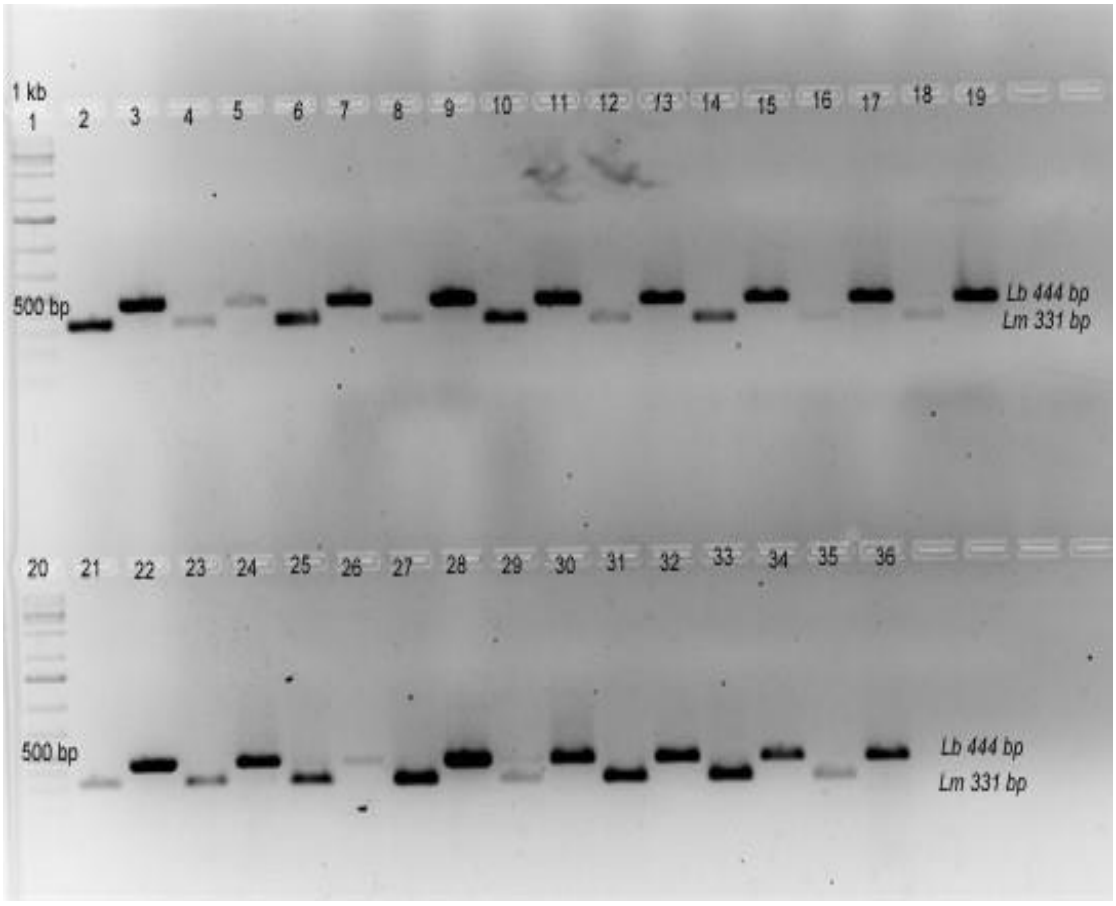

Figure 1: PCR amplification of *L. maculans* (*Lm*) and *L. biglobosa* (*Lb*) ITS sequences from Swedish samples using species-specific primers. *L. maculans* (Nz-T4), *L. maculans* (JN3) and *L. biglobosa* (G12-14) isolates were used as reference fungal strains. Upper lanes*:* **1**.1kb DNA ladder. ***2***. *Lm* (Nz-T4), ***3***. *Lb* (G12-14). **4, 6, & 8.** *Lm* sample 5. **5, 7, & 9**. *Lb* sample 5. **10, 12 & 14**. *Lm* sample 4. **11, 13 & 15**. *Lb* sample 4. **16 & 18**. *Lm* sample 2. **17 & 19**. *Lb* sample 2. Lower lanes*:* **20**. 1kb DNA ladder, **21**. *Lm* (JN3). **22**. *Lb* (G12-14). **23**. *Lm* sample 2. **24**. *Lb* sample 2. **25, 27 & 29.** *Lm* sample 3. **26, 28 & 30**. *Lb* sample 3. **31, 33 & 35**. *Lm* sample 1. **32, 34 & 36**. *Lb* sample 1.

3.2. Swedish *L. maculans* and *L. biglobosa* isolates showed similarity to *Leptosphaeria* isolates deposited in NCBI database

BLAST search results showed that the nucleotide sequences obtained from the amplification of ITS region and 5.8 rRNA gene using species-specific primers for *L. biglobosa* had 97.8% to 100% identity to the ITS 5.8 rRNA sequences of *L. biglobosa 'brassicae'* group strain Lb1215 (Accession number MK335622.1) whereas the sequences obtained from the amplification of ITS region and 5.8 rRNA gene using species-specific primers for *L. maculans* had 95.8% to 99.6 % identity to the ITS region and 5.8 rRNA sequence of *L. maculans* strain Alam10, a strain belongs to '*brassicae'* group (Accession number MT316111.1). In summary, the results revealed that all five samples gave amplification products for both *L. biglobosa and L. maculans* (Table 2).

Table 2: Blast search results of amplification products for five Swedish *L. maculans* and *L. biglobosa* isolates

Sample	Blast search results	Query cover	E-value	Percentage Identity
Sample 1 (*L. maculans*)	*L. maculans* strain Alam10	99%	1e-116	99.2%
Sample 1 (*L. biglobosa)*	*L. biglobosa 'brassicae' group strain Lb1215*	95%	0.0	99.2%
Sample 2 (*L. maculans*)	*L. maculans* strain Alam10	99%	1e-116	95.9%
Sample 2 (*L. biglobosa*)	*L. biglobosa 'brassicae' group strain Lb1215*	99%	4e-176	97.8%
Sample 3 (*L. maculans*)	*L. maculans* strain Alam10	99%	1e-105	95.5%
Sample 3 (*L. biglobosa*)	*L. biglobosa 'brassicae' group strain Lb1215*	99%	0.0	99.5%
Sample 4 (*L. maculans*)	*L. maculans* strain Alam10	96%	1e-120	99%
Sample 4 (*L. biglobosa*)	*L. biglobosa 'brassicae' group strain Lb1215*	95%	5e-166	96.9%
Sample 5 (*L. maculans*)	*L. maculans* strain Alam10	96%	3e-148	99.7%
Sample 5 (*L. biglobosa*)	*L. biglobosa 'brassicae' group strain Lb1215*	96%	0.0	100 %

3.3. Phylogenetic analysis and identification of SNPs

Sequence alignment indicated that the ribosomal RNA region incorporating the internal transcribed spacers and the 5.8S rRNA region is highly conserved among the *L. maculans* DNA and *L. biglobosa* DNA. However, few SNPs were observed when the Swedish *L. maculans* sequences were aligned with those of two reference strains of *L. maculans* (Nz-T_4) and *L. maculans* (JN3), as well as a reference strain of *L. biglobosa*, (G12-14) (Appendix Figure 2 and Appendix Figure 3). Phylogenetic analysis results showed that *L. biglobosa* isolates were well separated from *L. maculans.* All the *L. biglobosa* isolates clustered in one clade including the reference strain *L. biglobosa* (G12-14). However, *L. maculans* isolates showed genetic distance among samples (Figure 2).

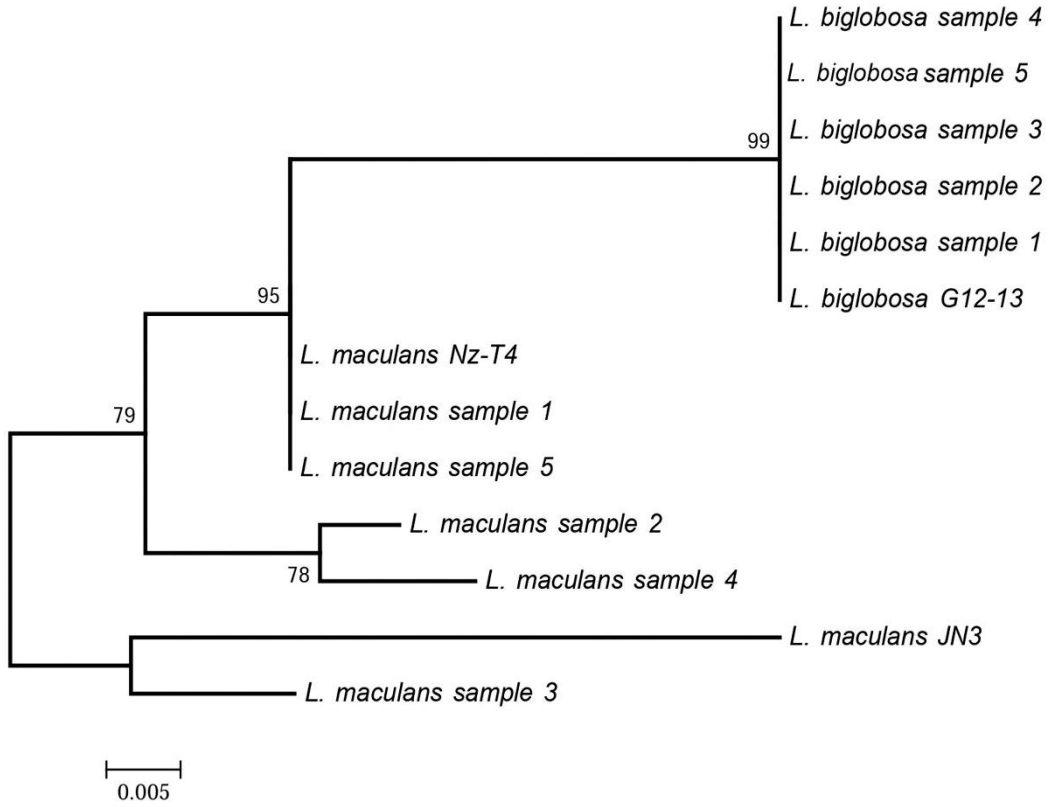

Figure 2: Phylogenetic analysis by using the maximum likelihood method with thirteen nucleotide sequences of internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. Bootstrap values (500 replicates) higher than 50% are indicated at the nodes. Bar = number of substitutions per site

3.4. DNA quantification

qPCR results showed that the amount of *L. maculans* and *L. biglobosa* DNA was significantly different in sample 2 and 3. Further, *L. biglobosa* DNA was higher in sample 2 whereas *L. maculans* DNA was higher in sample 3. There was not any significant difference between the amount of *L. maculans* and *L. biglobosa* DNA in sample 1, 4 and 5. The fungal DNA content detected in the samples ranged between 0.2 ng to 187.2 ng /100 mg infected stem for *L. maculans* and 1.6 ng to 93.9 ng /100 mg infected stem for *L. biglobosa* (Figure 3, Table 3). Standard error in some samples showed a large variation. Because the stem samples used in DNA extraction were very dry, purity and yield of the extracted DNA were reduced for some samples. Another possible reason could be pipetting errors.

Table 3: Amount of *L. maculans* and *L. biglobosa* DNA (ng) in 100 mg of dried stem sample

Sample	***L. maculans***	***L. biglobosa***
1	187.2	58.9
2	0.2	93.9
3	184.6	1.6
4	35.7	44.0
5	0.2	48.3

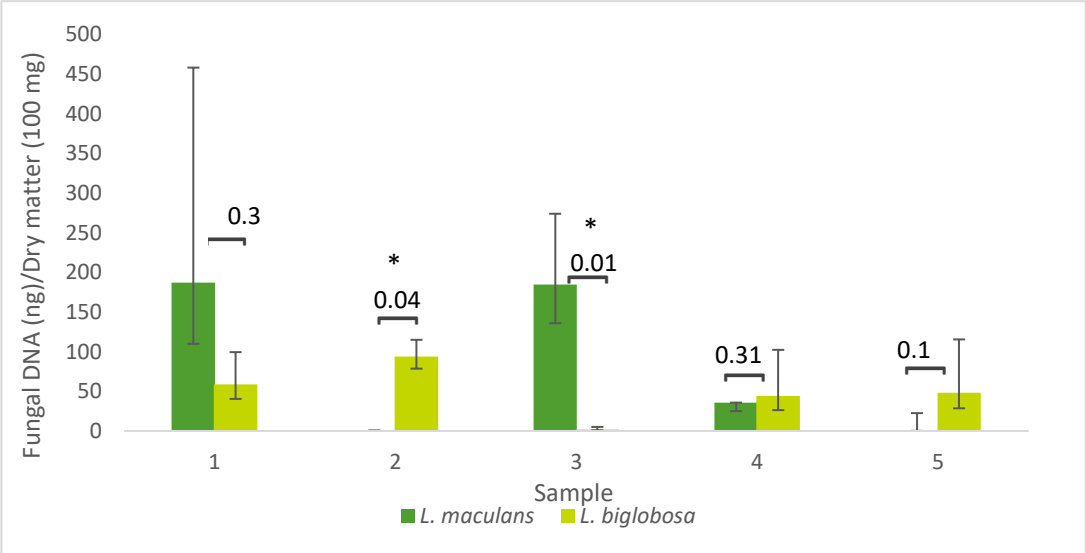

Figure 3: Absolute quantification of *L. maculans* and *L. biglobosa* DNA levels in five oilseed rape samples. Error bars indicate standard errors. The value above bars shows the corresponding p values obtained by Students's t-test ($p<0.05$ was used to measure significant difference).

3.5. Phenotypic evaluation of timothy in response to *Fusarium avenaceum*, *F. graminearum* and *Sclerotinia trifoliorum* infection

Sclerotinia trifoliorum did not induce any disease symptoms on the populations Switch, Kämpe and PKTT 2018 (Appendix Figure 3) and extended screening was not performed. Results of pathogenicity test under control environment conditions showed that two *Fusarium* species (*F. avenaceum* and *F. graminearum*) caused considerable infection of timothy seedlings with observable symptoms of retardation of growth, chlorosis of leaves and rotting of the roots and stem base. All the tested cultivars were susceptible to both *Fusarium* species, but severity of the infection differed among the genotypes. Damages due to *F. avenaceum* was higher than that of *F. graminearum.* PK TT 2018 showed highest partial resistance to *F. avenaceum* whereas SW 1023015 showed highest partial resistance to *F. graminearum.* Percentage of disease severity index 14 days post-inoculation in timothy populations are given below (Figure 4, Table 4).

Table 4: Disease severity index (DSI) % of timothy populations infected by two different *Fusarium* species

Timothy genotypes	***F. avenaceum* DSI**	***F. graminearum* DSI**
Switch[1]	88.2	-
Kämpe II	64.5	45
PK TT 2018	55.8	45
Ragnar	60	40
Rakel	75	75
Grindstad	75	62.5
Jonatan	100	60
Saga	92.5	17.5
SW 1023015	100	10
SW 1023030	80	15

[1] = *F. graminearum* isolate was received after monitoring the Switch cultivar

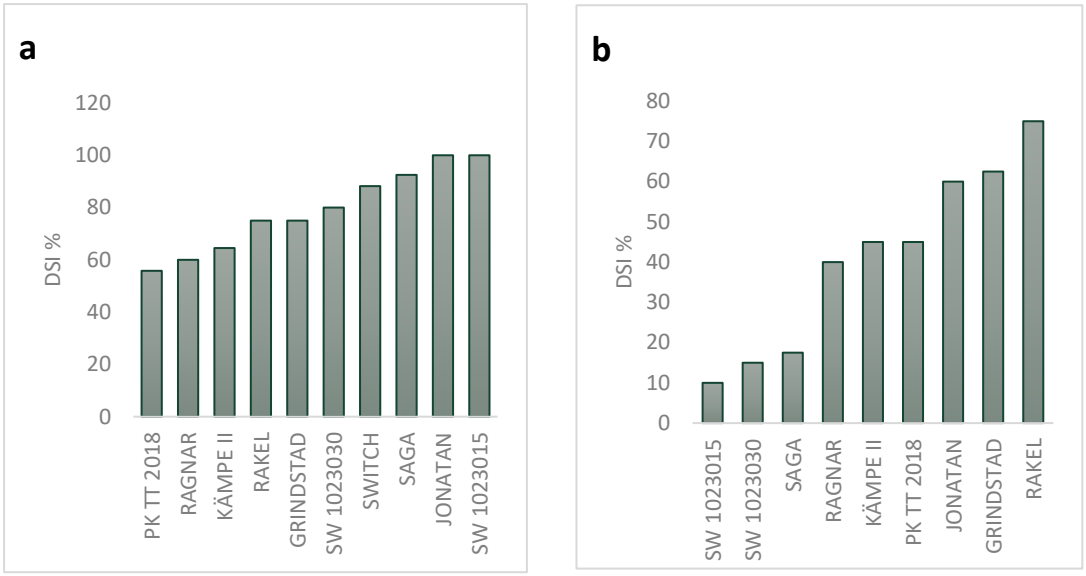

Figure 3: **a**) Disease severity indexes (%) of ten timothy populations in response to *F. avenaceum*. **b**) disease severity indexes (%) of nine timothy populations in response to *F. graminearum*.

3.6. Foliar phenotypes and root system phenotypes in response to *F. avenaceum* and *F. graminearum* infection

As mentioned in pathogenicity observation criteria (Table 1), dark brown to black colored roots were observed at 14 days post-inoculation. Areas of dead tissues were observed in crown and stem base (Figure 5). Chlorosis of the leaves took place gradually and finally turned into dark yellow or brown at 14 days post-inoculation (Figure 6).

Figure 5: Effect of *F. avenaceum* and *F. graminearum* on root and crown part of the timothy plants (cv. Jonatan) at 14 days post-inoculation. **a)** Root system and the basal part of plants grown under control condition **b**) Root system and the basal part of plants infected with *F. avenaceum* and **c**) Root system and the basal part of plants infected with *F. graminearum*.

Figure 6: Timothy foliar symptoms of *F. avenaceum* and *F. graminearum* inoculated plants of cv. Jonatan. **a**) Plants grown under control condition, **b**) Plants infected with *F. avenaceum* and **c**) Plants infected with *F. graminearum*.

4. Discussion

In this study, co-existence of *L. maculans* and *L. biglobosa* in the five Swedish oilseed rape stem samples was identified. All *L. maculans* isolates identified belonged to *L. maculans* 'brassicae' group whereas all *L. biglobosa* isolates belonged to the *L. biglobosa* 'brassicae' group. In two samples (sample 1 and 3), the amount of *L. maculans* DNA was greater than that of *L. biglobosa* DNA whereas in another set of samples (sample 2 and 4), the amount of *L. biglobosa* DNA was greater than that of *L. maculans* DNA. However significant difference between *L. maculans* and *L. biglobosa* was observed only in in sample 2 and sample 3.

Two *L. maculans* 'brassicae' isolates JN3 (v23.1.3) and Nz-T4 and one *L. biglobosa 'brassicae'* isolate, G12-14 were used as reference fungal strains in this study. JN3 is a reference sequence isolate of European origin (Rouxel et al., 2011) whereas Nz-T4 originates from New Zealand (Dutreux et al., 2018). *L. biglobosa* isolate G12-14 was isolated in France 2014. This isolate has the ability to produced typical symptoms of *L. biglobosa 'brassicae'* on oilseed rape plants in contrast to the previously sequenced *L. biglobosa* isolate B3.5 (Dutreux et al., 2018).

A study carried out by Grandaubert et al. (2014) based on alignment of 19 conserved proteins concluded that *Leptosphaeria* diverged around 73 million years ago from plant pathogens like *Cochliobolus, Pyrenophora* and *Alternaria* of the order Pleosporales. The study indicated that *L. maculans* and *L. biglobosa* divergence took place around 22 million years ago. According to parsimony and distance analysis, *L. maculans* is divided into two-sub clades and *L. biglobosa* into seven sub-clades, which correspond to the specific host plant and geographic origin (Zou et al., 2019). Among *L. maculans,* 'brassicae' is the genotype found on Brassica crops and likewise the *L. biglobosa* 'brassicae' group is the most common in most oilseed rape growing areas (Zou et al., 2019). Most often, *L. biglobosa* isolates are found in association with *L. maculans,* but *L. biglobosa* 'brassicae' is so far the only sub-species found in China (Zou et al., 2019).

BLAST search results showed that the ITS and 5.8s rRNA gene of all the *L. biglobosa* isolates from this study shared highest identity with *L. biglobosa*

'brassicae' group and phylogenetic analysis also clustered all the *L. biglobosa* isolates into a single clade. Even though the BLAST search showed that the nucleotide sequences of all *L. maculans* isolates from this study shared highest similarity with *L. maculans* strain Alam10 in the database, a strain that belongs to *L. maculans* 'brassicae' group, our phylogenetic analysis grouped them into three different sub-clades.

A study carried out by Kuusk et al. (2002) identified both *L. maculans* and *L. biglobosa* the infected leaf samples of Swedish winter oilseed rape. Even though the sample number in this study is very small, it also clearly demonstrated the presence of the two *Leptosphaeria* species in Sweden. There are several factors that impact genetic diversity. First, the capacity of crosses driven by the sexual spores. Temperature is one of the main factors that affect the maturation of pseudothesia (Toscano-Underwood et al., 2003). *L. biglobosa* prefers temperatures above 10 ˚C whereas pseudothesia maturation of *L. maculans* can take place at low temperatures. However, pseudothesia of both species mature at the same time at higher temperatures between 15 to 20 ˚C. In Skåne, the average temperature in winter is around 1 to 3 ˚C, while in spring it increases to 15 ˚C (https://www.worlddata.info/europe/sweden/climate-skane.php). However, climate models predict a future extension of up to 3 months in cultivation period reaching a vegetation period of 350 days and risk for more precipitation (https://www.smhi.se/klimat/framtidens-klimat/klimatscenarier/). Clearly such scenarios would promote establishment of *Leptospharia* species in Sweden. The *L. biglobosa* sub-species found on weedy species elsewhere may also become a problem in Sweden. It also highlights the importance of crucifer weeds that could act as hosts. In addition, *L. maculans* 'brassicae' strains harbour transposable elements in their genomes with capacity to further drive the genetic diversity in a population (Grandaubert et al., 2014).

Knowledge on diseases of forage grasses is limited in Sweden. However, cereals like small grains can be infected by several fungal pathogens and not least by *Fusarium* species (Karlsson et al., 2021). Several *Fusarium* species produce sexual spores and some do also produce hardy chlamydospores that can survive in soils.

Fusarium culmorum and *F. graminearum* produce chlamydospores (Goh et al. 2009) and (Ciotola et al. 2000). The latter species also forms both asexual and sexual spores which together drive the spread of the disease. The pathogen can cause disease on several plant parts, is prevalent in Sweden and rather common on cereals not least in the same crop rotation schemes as leys.

Fusarium avenaceum has been identified on red clover in Finland (Yli-Mattila et al. 2010) and is thought to cause root rot together with other *Fusarium* species in clover. Whether these *Fusarium* fungi have the ability of cross infection between grass and legume species has not been looked into before.

In this study, two *Fusarium* species originally isolated from wheat were found to cause disease symptoms on timothy similar as observed before on wheat (Yang et al., 2019) and red clover (Ylimaki, 1967). Under the present conditions, *F. avenaceum* caused more damage on timothy compared to *F. graminearum.* Cross pathogenicity between host species, is also a characteristic of the related soil-borne *F. oxysporum* (López-Orona et al., 2019).

Because *Fusarium* root rot and crown rot is caused of a *Fusarium* spp. complex, extra preventive measures should be taken during the field experiments in order to prevent mixed infections. The testing process can be carried out in well separated areas, and controlled infection can be carried out to observe damages caused by particular pathogen. During the infection process, continuous observations should be performed to ensure that the disease is due to the pathogen being tested. Microscopic observations and PCR-based tests can be carried out to confirm the identity of the pathogen attacking the host.

After field testing of *Fusarium*-damages in ley crops, next step could be work on improved levels of resistance since efficient chemicals commonly not are developed for soil-borne pathogens or for environmental reasons not an attractive choice.

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7. Appendix

Table 1: Composition of hydroponic solution

Stock solution	Amount in 1 l of hydroponic solution
KNO_3 (2.5 mM)	2.5 ml
$CaCl_2$ (1 mM)	1 ml
$MgSO_4$ (0.75 mM)	0.75 ml
KH_2PO_4 (0.75 mM)	0.5 ml
NH_4NO_3 (0.5 mM)	0.5 ml
Micronutrients (1 mM) (50 mM H_3BO_3, 10 mM $MnSO_4$ x H_2O, 1.5 mM $ZnSO_4$ x $7H_2O$, 1 mM $CuSO_4$ x $5H_2O$ and 0.58 mM Na_2MoO_4)	1 ml
Fe-EDTA (1 mM)	1 ml

Figure 1: Multiple sequence alignment of ITS region and 5.8 rRNA gene of *L. maculans* isolates from this study with reference fungal strains. *L. maculans* 1 and *L. maculans* 2 indicate *L. maculans* (isolate Nz- T_4) and *L. maculans* (isolate JN3), respectively.

```
L.biglobosa    1 AC-----------------------T-AT-TTGT-TTCC-TTGGTGGGCTTGCCTGCCA
samp 1         1 GTTT-------------TTTGCGTACT-AT-TTGT-TTCCTTTGGTGGGCTTGCCTGCCA
samp 2         1 CTGATTTACCCATGGTTTCTGCGTACT-AT-TTGT-TT-C-TTGGTGGGCTTGC-TGCCA
samp 3         1 GATTC-TACCCATGTTTTTTGCGTACT-AT-TTGT-TTCC-TTGGTGGGCTTGCCTGCCA
samp 4         1 TGTTT-----------TTAAGCGTACTTATTTTGTTTTCC-TTGGTGGGCTTGCCTGCCA
samp 5         1 TTTT--------------TTGCGTACT-AT-TTGT-TTCC-TTGGTGGGCTTGCCTGCCA

L.biglobosa   33 AAAGGACAATT-CAAACCACTT-GTAATTCGCAGTCAGCGTCAGTAACAATGTAATAATT
samp 1        45 AAAGGACAATT-CAAACCACTTAGTAATTCGCAGTCAGCGTCAGTAACAATGTAATAATT
samp 2        55 AAAGGACAATT-CAAACCACTC-GTAATT-GCAGTCAGCGTCAGTAACAATGTAATAATT
samp3         56 AAAGGACAATT-CAAACCACTT-GTAATT-GCAGTCAGCGTCAGTAACAATGTAATAATT
samp 4        49 AAAGGACAATTACAAACCACTCTGTAATC-GCAGTCAGCGTCAGTAACAATGTAATAATT
samp 5        43 AAAGGACAATT-CAAACCACTT-GTAATT-GCAGTCAGCGTCAGTAACAATGTAATAATT

L.biglobosa   91 ACAACT-TTCAACAACGGATCTCTT-GGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
samp 1       104 ACAACT-TTCAACAACGGATCTCTT-GGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
samp 2       112 ACAACT-TTCAACAACGGATCTCTTCGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
samp3        113 ACAACT-TTCAACAACGGATCTCTT-GGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
samp 4       108 AGAACTCTTCAACAACGGATCTCTA-GGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
samp 5       100 ACAACT-TTCAACAACGGATCTCTT-GGTTCTGGCATCGATGAAGAACGCAGCGAAATGC

L.biglobosa  149 GATAAGTAGTGTGAATT-GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
samp 1       162 GATAAGTAGTGTGAATT-GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
samp 2       171 GATAAGTAGTGTGAATT-GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
samp3        171 GATAAGTAGTGTGAATT-GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
samp 4       167 GATAAGTAGTGTGAATTCGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
samp 5       158 GATAAGTAGTGTGAATT-GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG

L.biglobosa  208 CCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTCTGCT
samp 1       221 CCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTCTGCT
samp 2       230 CCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTCTGCT
sam3         230 CCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTCTGCT
samp 4       227 CCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTCTGCT
samp 5       217 CCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTCTGCT

L.biglobosa  268 TGGTGTTGGGTGAATGTTCTCTGTGCTTGCGCAGACTGGACTCGCCTGAAAACAATTGGC
samp 1       281 TGGTGTTGGGTGAATGTTCTCTGTGCTTGCGCAGACTGGACTCGCCTGAAAACAATTGGC
samp 2       290 TGGTGTTGGGTGAATGTTCTCTGTGCTTGCGCAGACTGGACTCGCCTGAAAACAATTGG-
samp3        290 TGGTGTTGGGTGAATGTTCTCTGTGCTTGCGCAGACTGGACTCGCCTGAAAACAATTGG-
samp 4       287 TGGTGT-GGGTGAATGTTCTCTGTGCTTGCGCAGACTGGACTCGCCTGAAAACAATTGGC
samp 5       277 TGGTGTTGGGTGAATGTTCTCTGTGCTTGCGCAGACTGGACTCGCCTGAAAACAATTGGC

L.biglobosa  328 AGCCGGCATATTGGCCTGGAGC
samp 1       341 AGCCGGCA----------T
samp 2       349 ----------------C
samp 3       349 ----------------C
samp 4       346 AGC-------------C
samp 5       337 AGCCGGCATAT---------TG
```

Figure 2: Multiple sequence alignment of ITS region and 5.8 rRNA gene of *L. biglobosa* isolates with reference fungal strain *L. biglobosa* isolate G12-14.

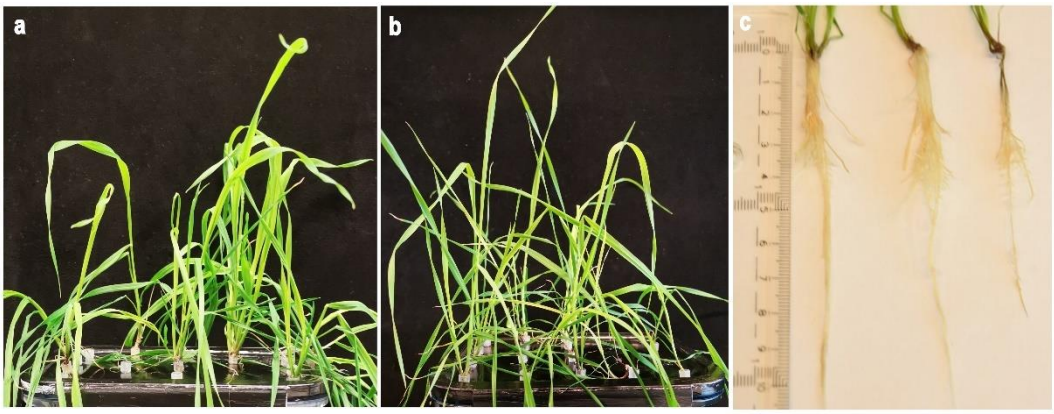

Figure 3: Timothy foliar and root system phenotypes of *S. trifoliorum* inoculated plants of cv. Switch. a) Plants grown under control condition, b) Foliar phenotypes of plants inoculated with *S. trifoliorum.* c) Root system phenotypes of plants inoculated with *S. trifoliorum* at 14 days post inoculations.

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