Image analysis of co-infection of different stem rust races

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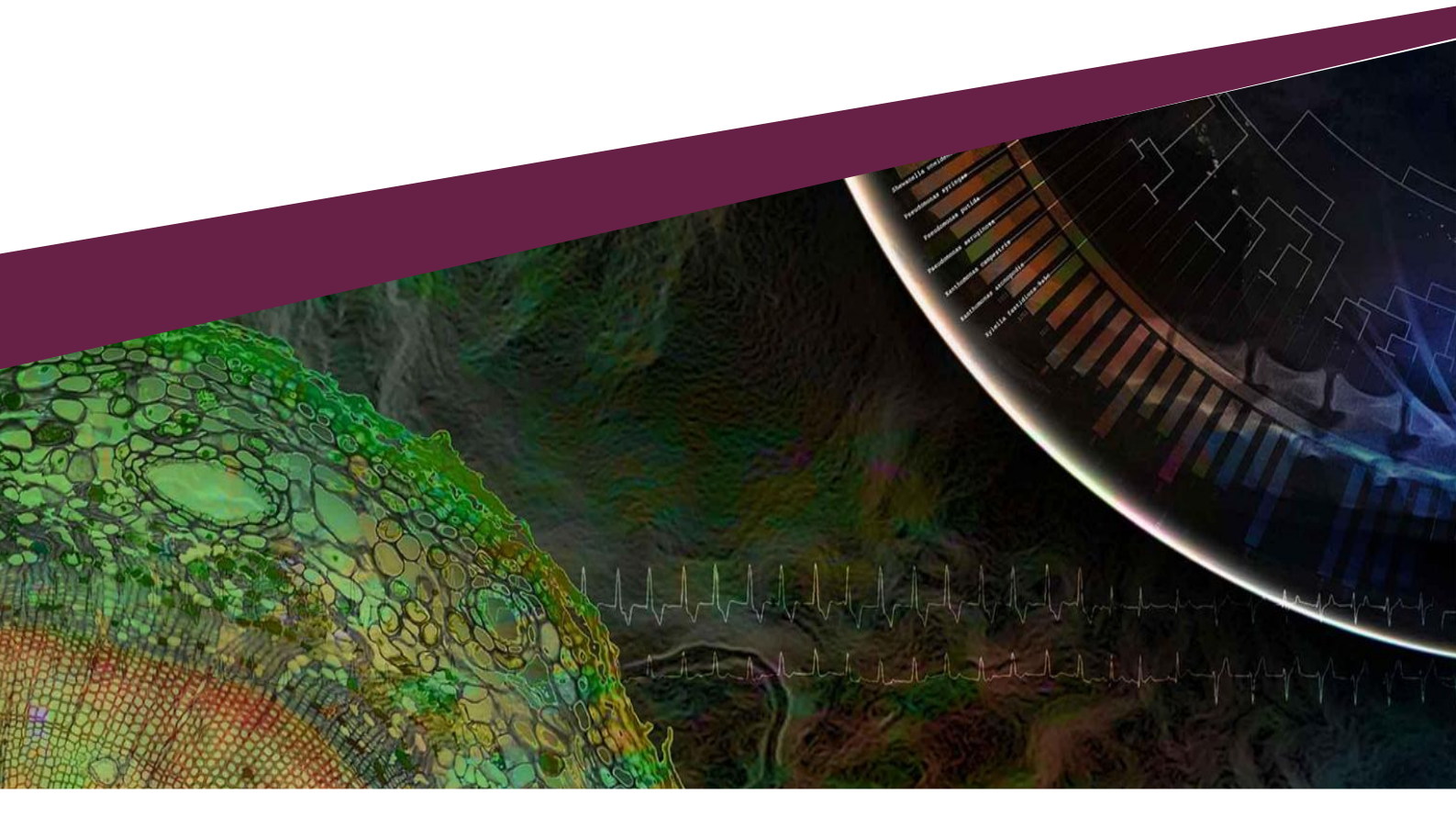
Publisher Forest mycology and plant pathology
2026



Co-infection of different stem rust races

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Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Master thesis in Biology
Course code:	EX0898
Programme:	Agriculture Programme - Soil and Plant Sciences
Course coordinating dept:	Department of Forest Mycology and Plant Pathology
Publisher:	Swedish University of Agricultural Sciences
Year of publication:	**2026**
Place of publication:	**Uppsala**
Copyright:	All featured images are used with permission from the copyright by owner.
Keywords:	Image analysis, co-infection, *Puccinia graminis* f.sp *tritici*, Naïve-bayes, wheat

Sammanfattning

Svartrost, som orsakas av *Puccinia graminis* f.sp *tritici* (Pgt), är på väg att återigen bli ett allvarligt problem inom veteproduktionen. Denna studie undersöker effekten av kombinerad infektion av fyra olika Pgt raser på vete samt möjligheten att utveckla en metod för bildanalys av sjukdomsangrepp. Fyra Pgt isolat, insamlade i Uppland 2017, med olika komplexitet av virulensgener, inokulerades ensamma och i kombination med varandra på mottagliga och toleranta vetesorter. Angreppen bedömdes både okulärt och med hjälp av Naïve-Bayes maskininlärning bildanalys från PlantCV.

Resultaten visade att mindre komplexa isolat, särskilt SE279a_17, orsakade tidigare och allvarligare symptom än isolat med högre komplexitet. Detta antyder att det finns en metabolisk kostnad för patogenen att bära på flera virulensgener. Kombinerad infektion gav inte konsekvent större angrepp, vilket indikerar att det finns en komplex interaktion mellan raserna.

Denna studie visar att enbart hög komplexitet av virulensgener inte räcker för att förutse aggressivitet och påvisar vikten av interaktionen mellan värden och patogenen. Bildanalys gav en säker bedömning av symptomen för sjukdomen och är ett verktyg för framtida resistensforskning och växtförädling.

Nyckelord: Bildanalys, kombinerad infektion, *Puccinia graminis* f.sp *tritici*, Naïve-bayes, vete

Abstract

Stem rust, caused by *Puccinia graminis* f. sp. *tritici* (Pgt), is a re-emerging major threat to wheat production. This study examined the effects of co-infection of four different Pgt races on wheat and the possibility to develop a method for image analysis of disease severity. Four Pgt isolates, sampled in Uppland in Sweden 2017, with different virulence gene complexity, were inoculated alone and in combinations on susceptible and resistant wheat varieties. Disease severity was assessed with manual ocular scoring and Naïve-Bayes machine learning image analysis from PlantCV.

Results showed that less complex isolates, particularly SE279a_17, caused earlier and more severe symptoms than more complex isolates, suggesting a fitness cost following a higher number of virulence genes. Co-infection did not consistently increase disease severity, indicating complex interactions between races.

This study shows that high virulence gene complexity alone does not predict aggressiveness, highlighting the importance of host-pathogen interactions. In

addition, the method image analysis provided accurate disease assessment, and offers a tool to screen for rust resistance in both research and for breeding purposes.

Keywords: Image analysis, co-infection, *Puccinia graminis* f.sp *tritici*, Naïve-bayes, wheat

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Abbreviations

Pgt	*Puccinia graminis* f.sp *tritici*
f. sp	*formae speciales*

1. Introduction

1.1 Background and hypothesis

The aim of this project was to develop and test a method to evaluate disease severity on leaves using image analysis. The method was developed to investigate the effect of co-infections by different races of stem rust on wheat. The aggressiveness of the fungal pathogen *Puccinia graminis* on wheat inoculated as a single race or in combination with other races was evaluated.

Stem rust on wheat (*Triticum aestivum*) is caused by a basidiomycete fungus, *Puccinia graminis* f. sp. *tritici* (Pgt). *Puccinia graminis* is a heteroecious fungus, meaning that it requires two hosts to complete its sexual life cycle. Pgt infects grasses as its telial host and barberry (*Berberis* spp.) as its aecial host. Stem rust is primarily considered a warm climate disease and more prevalent in regions where summer temperatures regularly exceed 25°C. At the same time, it can cause serious damages on susceptible wheat varieties over large geographical regions (Leonard & Szabo 2005). Heavy infection reduces nutrient flow to the heads, which results in shrivelled grain and lower yield. The stems are also weakened, which makes them prone to lodging (Leonard & Szabo 2005).

Stem rust has historically been a feared disease in all wheat growing regions. There are records of Roman festivals with rituals and sacrifices to appease the rust gods and the bible has several rust references (Leonard & Szabo 2005). However, stem rust was considered insignificant in Europe for over 60 years (Singh et al. 2015). The last epidemic in Sweden occurred in 1951 and resulted in a national yield loss of 9-33% in wheat (Åkerman et al. 1952). The control of stem rust during this long time was based on the removal of the alternate host *Berberis* spp. to prevent *P. graminis* from completing its sexual life cycle in combination with the introduction of resistant varieties that were a result of successful efforts in identifying and breeding new wheat cultivars with race specific resistance to *P. graminis*. Due to the reduced genetic diversity in Pgt populations caused by the inability to complete its sexual life cycle, these genes have successfully protected wheat from stem rust for a long time (Jin 2011).

After the law for eradicating *Berberis* spp. was repelled in Sweden in the late 20th century, berberis bushes have become more common in the landscape. Interestingly, the UK have initiated programs to mass plant barberry bushes in order to help the endangered barberry carpet moth (Lewis et al. 2018). The wide spread occurrence of berberis has given Pgt better possibilities to complete its life cycle and as a result, new strains virulent to existing resistance genes have developed (Lewis et al. 2018). Lewis *et al* (2018) found after inoculating 57 wheat varieties, that a new race of Pgt, designated UK-01, was virulent on 37 of these varieties. They determined that UK-01 belonged to the TKTTF race previously found in Germany 2013.

In Sweden a similar test was conducted on commercially available varieties. 37 wheat and 3 barley varieties were tested with a Pgt population collected from the severe outburst of stem rust in Uppland, Sweden in 2017 (*Nr 21. Vete- och vårkornsorters mottaglighet för den nya populationen av svartrost i Sverige_Uppsala [2]* n.d.).Another Pgt race, TTRTF, virulent to 70% of commercial wheat varieties in Europe have been identified in northern Europe. It has also been found in Sweden in 2021 (Patpour et al. 2023).

Resistances against rusts are primarily qualitative and controlled by specific genes. This suggests that clonal, asexual reproduction on a host allows for rapid multiplications of virulent isolates on that particular host. Sexually recombining populations select for isolates that will be able to infect both the aecial and telial host (Ali et al. 2010). Based on previous results, we know that the number of virulence genes carried by different pathogen genotypes varies within sexual populations. Some successful isolates have few virulence genes while other isolates carry several. It has been shown for *P.striiformis* that multiple virulences imposed a substantial fitness cost in the absence of the corresponding reistance genes(Bahri et al. 2009).

Thus, it can be hypothesised that when several different isolates in a sexual population of *Puccinia graminis* are combined, the resistance pattern becomes more quantitative. In addition, it is thought that there is a fitness cost in sexual populations caused by the ability to infect both the aecial and the telial hosts (Bahri et al. 2009). However, this could be compensated by positive interaction between strains as they together "open up" the plant defence, as co-infections of strains from sexual populations that individually carry few virulence genes may create just as severe epidemics as hyper virulent clonal lineages. This pattern is due to the co-speciation pattern (De Vienne et al. 2013) between different groups of *P. graminis*, following the speciation of the grass hosts and concurrently, a plasticity of host barriers within *P. graminis* (Patpour et al. 2022).

Therefore, in this thesis I examine if this is partly compensated by positive interaction between strains as they may "open up" the defence and can create just as severe epidemics as hyper virulent clonal linages.

1.2 Disease symptoms & life cycle

The pathogen *P. graminis* can cause stem rust on 365 different cereals and grasses (Leonard & Szabo 2005). Based on the host they are classified as different *formae speciales* (f. sp), e.g *Puccinia graminis* f.sp *secalis* infects rye and f.sp *avenae* infects oats (Berlin et al. 2012). The disease symptoms mainly develop on stems and leaf sheaths with small chlorotic flecks as first visual symptoms a few days after first infection. After 8-10 days after infection a mass of brown/red urediniospores erupts from the epidermis.

The urediniospores are wind-dispersed and continue to spread and infect the main hosts until late in the summer in temperate regions when temperatures start to drop. *Puccinia graminis* then start to produce two celled teliospores, which are the resting spores of *Puccinia graminis*. Meiosis begins shortly after, before the teliospore enters dormancy, resulting in four haploid nuclei (two of each mating type, + and -) per teliospore cell. Within each cell the two haploid nuclei fuse in early teliospore maturation forming one diploid cell per teliospore. These teliospores are black in colour while the urediniospores are brown. On cereals they remain dormant on infected straw until spring when they germinate in unison with the budding on berberis.

A hyphae called the promycelium grows from two teliospores (Leonard & Szabo 2005). The basidiospores are ejected from the promycelium and carried by wind to the alternate host *Berberis* spp. (Bueno-Sancho et al. 2021). The basidiospors infect the leaves and form the pycnium which produce pycniospores, these spores are the male gametes. From the top of the pycnium structure grows hyphae which serve as the female gametes. When a pycniospore from one mating type, +, contacts the hyphae from another mating type, -, a fusion tube forms between them to allow the haploid nuclei from the pycniospore to migrate through the hyphae towards the base of the pycnium to the protoaecium. Here the dikaryotic stage is established and the aecium is formed which produce dikaryotic aeciospores that infect the main grass hosts (Leonard & Szabo 2005).

1.3 Race definition

Within each *formae speciales* the individual *P. graminis* isolates are divided into physiologic races. An isolate is tested on a differential set with a specific set of resistant wheat varieties containing a collection of set resistance genes. The races are first designated by a three-letter code and additional letters depending on which resistance genes the isolate is virulent against. A race that is designated with letters

earlier in the alphabet than other races indicates that it is more virulent than the latter (Roelfs 1988).

1.4 Stem rust resistance in cereals

Plant resistance to pathogens provides farmers with an additional tool to combat disease and yield loss in their crops. However, standardization and intensification of modern agriculture as well as Pgt's high evolutionary potential has generally led to recurring boom-and-bust cycles i.e rapid pathogen adaptation to new resistance genes and combination of those.

There are over 40 distinct genes for resistance to different races of Pgt (McIntosh et al. 1995), and for each resistance gene there is a matching avirulent gene in the pathogen (Leonard & Szabo 2005). This avirulent gene is what the plants need in order to recognise the pathogen and initiate a resistance response.

Plant disease resistance in commercially grown varieties is often based on monogenic resistance with a major resistance gene, conferring qualitative resistance making the plant almost immune to the disease (Dormatey et al. 2020) to parts of the pathogen population. This puts a very high selection pressure on the pathogen leading to a rapid resistance breakdown as compared to polygenic quantitative resistance. By combining several resistance genes from different sources in one variety, so called gene pyramiding, breeders are able to produce varieties with a long-term and durable resistance a single pathogen or pest (Malav et al. 2016).

1.5 Image analysis

Assessment and quantification of disease severity caused by a pathogen is usually a time-consuming process, which also require great expertise by the assessor to produce an accurate assessment and conclusion. Accurate leaf analysis is critical in agricultural research and crop management, as leaves are primary indicators of plants exposure to stress or disease.

This requirement for disease assessment and phenotype evaluation has led to the development of computer models and machine learning scripts capable of evaluating disease severity caused by many different pathogens from images of the plant and disease symptoms. This gives researchers a tool to quickly and accurately evaluate large quantities of plant material for favourable traits in plant breeding programs (Gehan et al. 2017). The models learn patterns from large datasets, making them highly adaptable to complex agricultural scenarios with differences in lighting, background texture, and between plant varieties (Ferentinos 2018).

With advancements in computer vision and machine learning, researchers can now accurately detect and quantify diseases, estimate yield, and classify plant species using image analysis (Kamilaris & Prenafeta-Boldú 2018).

2. Materials and Methods

To examine the aggressiveness of four isolates of *P. graminis*, alone and in combination, an inoculation experiment was set up in a controlled environment. The isolate's aggressiveness on four spring wheat varieties were determined.

2.1 Isolates

The four isolates used in this experiment originated from a population found in Uppland in Sweden in 2017 (Nr 21. Vete- och vårkornsorters mottaglighet för den nya populationen av svartrost i Sverige_Uppsala [2] n.d.). Sexual reproduction was indicated by a high number of genotypes compared to sampling size, linkage disequilibrium close to zero and high genotypic diversity indexes. (Patpour et al. 2022).

The isolates, as listed in table 1, were phenotyped by Merham Patpour at Global Rust Reference Centre, Aarhus university, Denmark (Patpour et al. 2022). Each isolate was then given a race classification for this experiment depending on their number of known simple, intermediate and complex virulence genes,. The isolates were chosen for this experiment due to their difference in number of virulence genes.

Table 1. Isolate id, pathotype, race classification and virulence genes. The virulence genes Sr5, Sr8a, Sr9a, Sr17, SrMcN were fixed between the isolates

Isolate ID	**Isolate id***	**Race pathotype**	**Race classification**	**Virulence genes**
SE279a_17	279	LFCLC	Simple	*Sr9g*
SE280e_17	280	MFCNC	Intermediate	*Sr7b, Sr9g, Sr10*
SE283c_17	283	QFCSC	Intermediate	*Sr9d, Sr9g, Sr10, Sr21*
SE287e_17	287	RKHNF	Complex	*Sr6, Sr7b, Sr9b, Sr9g, Sr10, Sr21, Sr38*

**Isolates will be referred to the assigned isolate id for the remainder of the thesis*

2.2 Spore multiplication

The spring wheat variety Diskett was sown in pots (10x10 cm) placed in plastic trays. In each pot, between 10 and 20 seeds were sown in peat soil and placed in growth chambers with a photoperiod of 16 hours light and 8-hour dark and with a temperature range of 20-22°C during the day and 18-20°C during the night and a relative humidity of <80%. After 8 days the seedlings were inoculated with the different Pgt isolates, with one isolate per pot. Spores were suspended in 1.7 ml of water and a drop of Tween 20 and brushed on the seedlings using small paint brushes.

Each pot was placed on blue tray cups filled with hot water and sealed with clear plexiglass tubes and another blue tray cup on top. This was done to achieve 100% humidity. All pots were placed in a dark room for a minimum 24 hours at room temperature. Then, the blue tray cup and plexiglass tubes were removed and the inoculated plantlets moved to a growth chamber with a photoperiod of 16 hours and a temperature of 20-22 °C and relative humidity of <80%. The spores were harvested by shaking the plants over aluminium foil and poured into a 2 ml screw cap tube and placed in a desiccator for a few days and then stored in 7°C until future inoculations.

2.3 Variety selection

In a first experiment, the two wheat varieties Morocco and Line E were selected due to their universal susceptibility to stem rust.

In an additional experiment, three wheat varieties (Chinese spring, Fielder and Bob white) were inoculated with the four Pgt races (Table 2). The plants were scored 0-4 (Table 2). A score of 2 or below suggests that the variety is resistant to that isolate and 3 or above as susceptible (Patpour et al. 2022).

Table 2. Results from testing the resistance of varieties Bob white and Fielder. A 0-2 (green) score indicates resistance, a score of 3-4 (orange) indicates susceptibility.

ID	Race	Bob white	Chinese spring	Fielder
SE279a_17	LFCLC	3+ / 4-	3+ / 4	0;
SE280e_17	MFCNC	1 / 2	3+ / 4	1
SE283c_17	QFCSC	3 / 4	4	0; / 1

SE287e_17	RKHNF	1	4	2

2.4 Experiment

Two spring wheat varieties universally susceptible to Pgt (Morocco and Line E) and two resistant or partially resistant to Pgt (Fielder and Bob White) were used to conduct this experiment. For each variety, the four isolates were inoculated in the following combinations, intermediate-intermediate, simple-intermediate, simple-complex, and intermediate-complex as well as the isolates by themselves as positive control and one treatment of each variety inoculated with water as negative control (Figure 1). Spore weight for the first experiment was measured in mg while spore weight was measured in µg for the second experiment. Spore concentration for the first experiment was 1000 times higher than the spore concentration for the second experiment (Table 3).

For each biological replication five seeds were seeded along one edge of a pot filled with peat soil. At the two-leaf stage, the primary leaf of at least three plants per pot were threaded through a plastic coated paper plate in order to provide a stable platform to inoculate the spores on to the leaf. A pair of two lines were drawn on the plates 1.25 cm apart and 2 cm separating the pair; this was done to create a repeatable inoculation area for each leaf (Figure 1). The two areas were designated A and B as seen in figure 1.

The seedling pots were incubated the same way as for spore production and after 24 hours they were moved to growth chambers with a photoperiod of 16 hours light and 8-hour dark and with a temperature range of 20-22 °C during the day and 18-20 °C during the night and a relative humidity of <80%. The experiment was conducted twice with wheat varieties Morocco, Line E and Fielder in the first experiment, and with wheat varieties Morocco, Bob White and Fielder in the second experiment.

Table 3. Inoculated spore weight and concentration for the second replicate

Isolate	**Spore weight (µg)**	**Spore concentration spores/ml**
SE279a_17	62	110 000
SE280e_17	51	80 000
SE283c_17	57	110 000
SE287e_17	53	100 000

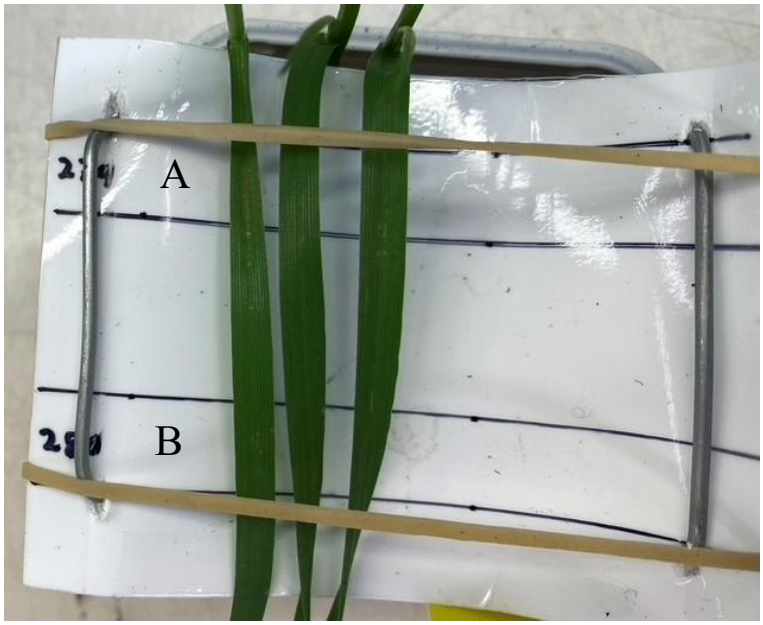

Figure 1. Experiment layout. Example figure shows race 279 inoculated between the black lines at the top (A) and race 280 inoculated between the black lines at the bottom (B) of the platform

2.5 Disease assessment

Latency period and disease severity were used to assess the aggressiveness of each isolate on the different wheat varieties. Latency period was determined as the time (in days) from infection to the appearance of sporulating lesions. The first experiment was visually scored on a scale of 0-4 (Stakman et al. 1962), with leaves only showing chlorotic symptoms scored as 0.5. after 16 days. The second experiment was also visually scored at 10, 12 and 14 days after inoculation (DAI).

Both experiments were harvested for image analysis after 16 DAI by taking photos (Figure 2) of the inoculated leaves, and visually scored on a scale of 0-4 (Stakman et al. 1962), with leaves only showing chlorotic symptoms scored as 0.5.

2.6 Image analysis

To phenotype the disease severity for the different races, alone and in co-inoculations, the three infected leaves were cut from the plant and taped to a A4 paper sheet marked with the wheat variety and isolate interaction (Figure 2).

Photos of each set of leaves were taken and the picture with the sharpest focus for each interaction was selected to be used for the image analysis. The inoculated area of the A and B isolate on the pictures were cropped using ImageJ to a set area of 123*270 pixels (Figure 3). The leaf area was assumed to be the same for both

wheat varieties. The cropped pictures were analysed using the software PlantCV (Gehan et al. 2017) with a Naïve Bayes script (*Naive Bayes* n.d.). This script counts the number of pixels from pustules, chlorotic and healthy tissue respectively from photos of the infected leaves (Figure 3). By individually dividing the number of pixels from pustules with the number of pixels from healthy tissue and the number of pixels from chlorotic tissue with the number of pixels from healthy tissue, a pustule index and chlorotic index was calculated from the raw data from the Naïve Bayes script.

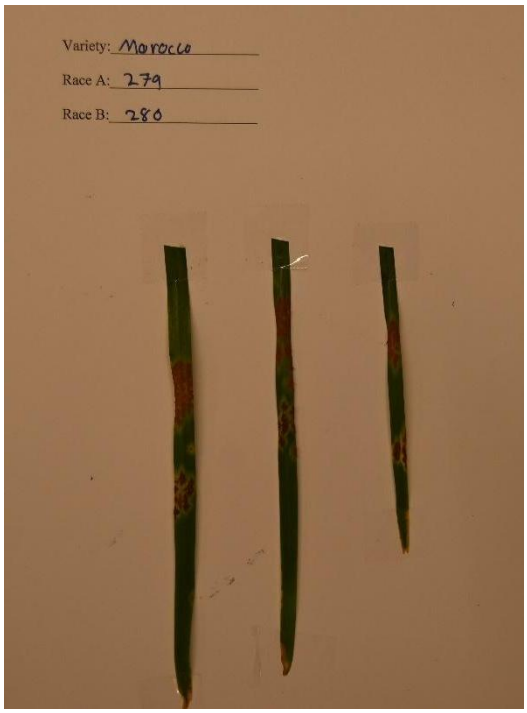

Figure 2. Picture of infected leaves from the susceptible wheat variety Morocco inoculated with isolates 279 and 280.

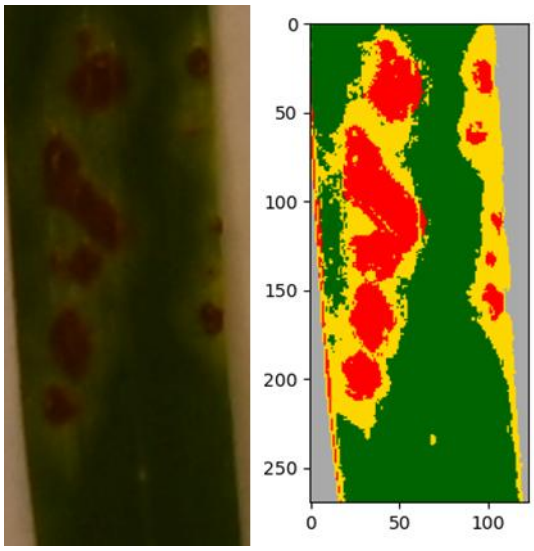

Figure 3. Cropped picture (left) of a disease area on a leaf and identified pixels of pustules (red), chlorosis (yellow) and healthy tissue (green) from the Naïve bayes script (right).

2.7 Statistical analysis

The results from the image analysis were analysed for normality using visual methods of histograms and normal Q-Q plots and the Kolmogorov-Smirnov test (Lopes 2011). The data was found to not be normally distributed, and thus the data was analysed with non-parametric methods of robust ANOVA and robust pairwise post-hoc test. Statistical analyses were done in R 4.2.2 (R core team 2022).

3. Results

The experiment was conducted twice with some differences. The first difference was that the susceptible wheat variety Line E was replaced in the second experiment for the semi-resistant wheat variety Bob White. The second difference was the inoculated spore concentration. In the first experiment, the plants were inoculated with a high concentration mixture of 0.5-0.6 mg of spores suspended in 1 ml of water, while the second experiment was inoculated with 0.5-0.6 µg of spores suspended in 1 ml of water. The third difference is the disease scoring. The first experiment was only scored after 16 DAI, while the second replicate was scored at multiple times: 10, 12, 14 and 16 DAI.

In the test for variety resistance, Fielder developed disease symptoms with a score of 2 and below for all four races. Bob White was found to be resistant to races 280 and 287 but susceptible to races 279 and 283 (Table 2). Isolates 279 and 280 generally developed chlorotic symptoms on all the tested varieties earlier than isolates 283 and 287, however there are no indication of any difference for the period from appearance of chlorotic spots to the emergence of pustules between the isolates (Table 4).

Table 4. Days after inoculation (DAI) and latency period in days for the two replications of the experiment.

Variety	Isolate	Chlorosis DAI	Latency period (DAI	Overall disease score*
Morocco exp 1	279	7	10	3.2
	280	7	10	3.2
	283	11	13	0.8
	287	10	13	2
Fielder exp 1	279	8	10	1.2
	280	8	13	0.6
	283	14	16	0.3
	287	13	14	0.5
Line E exp 1	279	7	10	3.1
	280	7	10	1.8
	283	9	10	2.2
	287	11	13	2.3
Morocco exp 2	279	7	10	2.1
	280	7	8	1.8
	283	7	10	2.1
	287	7	10	1.9
Fielder exp 2	279	7	10	0.8
	280	7	14	0.6
	283	8	12	0.4
	287	8	12	0.6
Bob White exp 2	279	8	10	0.8
	280	10	12	0.4
	283	7	10	0.9
	287	10	12	0.3

**Overall disease score was calculated as an average score from the 21 repetitions for each of the isolates.*

3.1 Experiment #1

The plants were scored at the time of harvest at 16 DAI. Robust ANOVA showed a significant difference ($p<0.05$) between the disease score means of the isolates (Figure 4). Robust pairwise post-hoc test showed that isolate 279 was significantly differentiated from isolates 280, 283 and 287 on disease score. Isolate 280 was significantly differentiated from isolate 283 (Figure 4).

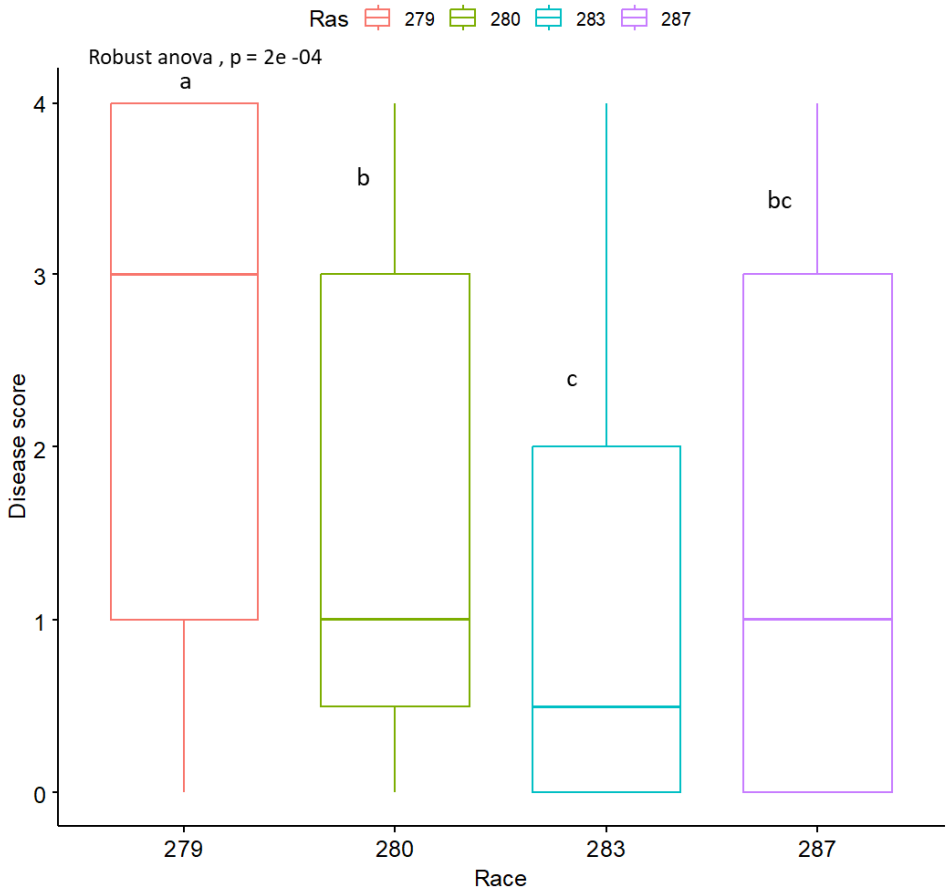

Figure 4. Disease score at 16 DAI for each isolate from experiment repeat 1. Robust ANOVA indicates a significant difference between the means at $p<0.05$. Different letters indicate a significant difference ($p<0.05$) by robust pairwise post-hoc test.

The disease score for each wheat variety shows that isolate 279 and 280 were scored significantly higher than isolate 283 and 287 on the susceptible variety Morocco. On the susceptible variety Line E the only significant difference was found between isolates 279 and 280 . For the resistant variety Fielder, the isolates were generally scored lower and isolates 279 and 280 were significantly different from isolate 283 (Figure 5).

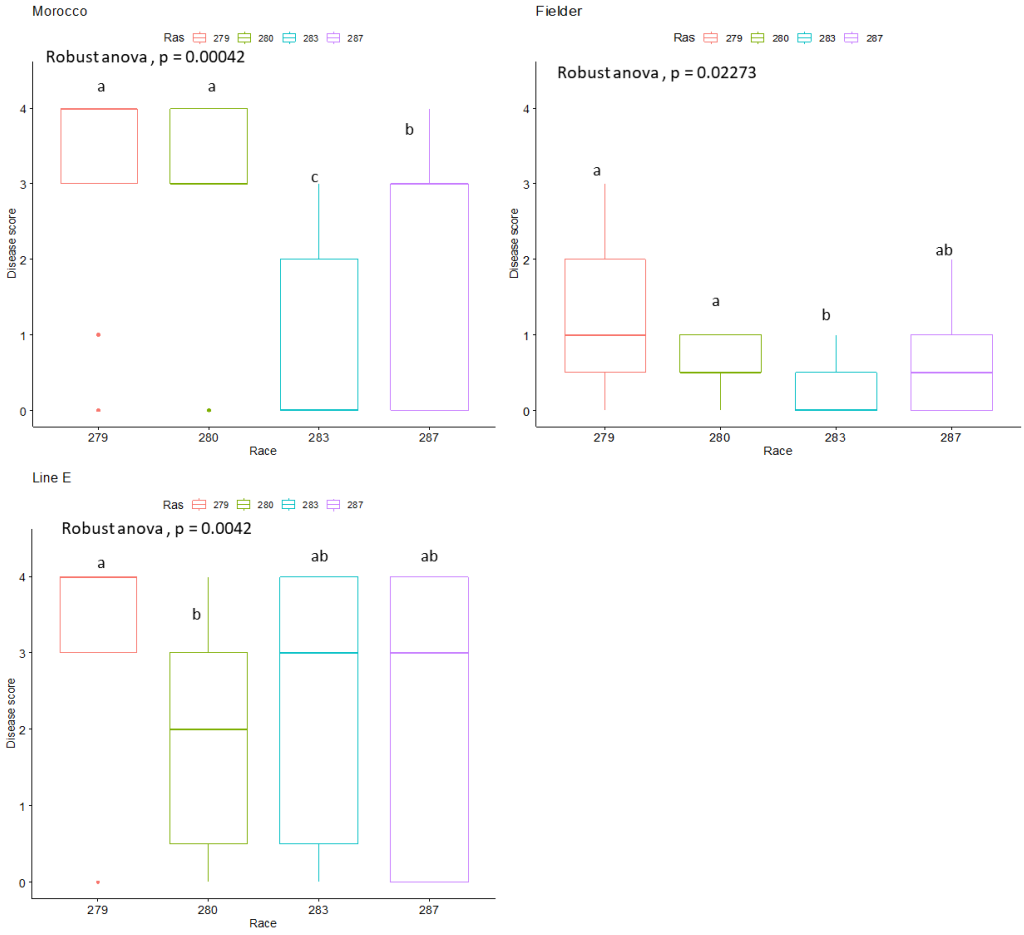

Figure 5. Disease score at 16 DAI for each isolate separated for each wheat variety from experiment repeat 1. Robust ANOVA indicates a significant difference between the means at $p<0.05$. Different letters indicate a significant difference ($p<0.05$) by robust pairwise post-hoc.

From the image analyses, the pustule index, when separated into the three wheat varieties showed significant differences between the isolates infecting the susceptible wheat varieties Morocco and Line E as seen in table 5 indicated by the p-value. Isolates infecting the resistant wheat variety Fielder showed no differences between their means (Table 5; Figure 6). Isolate 279 on Morocco had a significantly higher pustule index than isolates 283 and 287 (Figure 6). Isolate 283 had significantly lower pustule index than the other isolates on Morocco (Figure 6). On Line E, isolate 279 had significantly higher pustule index than isolates 280, 283 and 287. Isolate 280 had significantly lower pustule index than isolate 279 and 283 (Figure 6).

Table 5. Pustule index for the different isolates inoculated on the varieties Fielder, Morocco and Line-E. Different letters indicate a significant difference ($p<0.05$) between the isolates according to robust pairwise post-hoc test.

Isolate	p-value*	Robust post-hoc
Fielder		
279		a
280	0.104	a
283		a
287		a
Morocco		
279		a
280	0.019	ab
283		c
287		b
Line E		
279		a
280	0.007	b
283		c
287		bc

**p-value represents the significant differences between the isolates grouped by the three wheat varieties.*

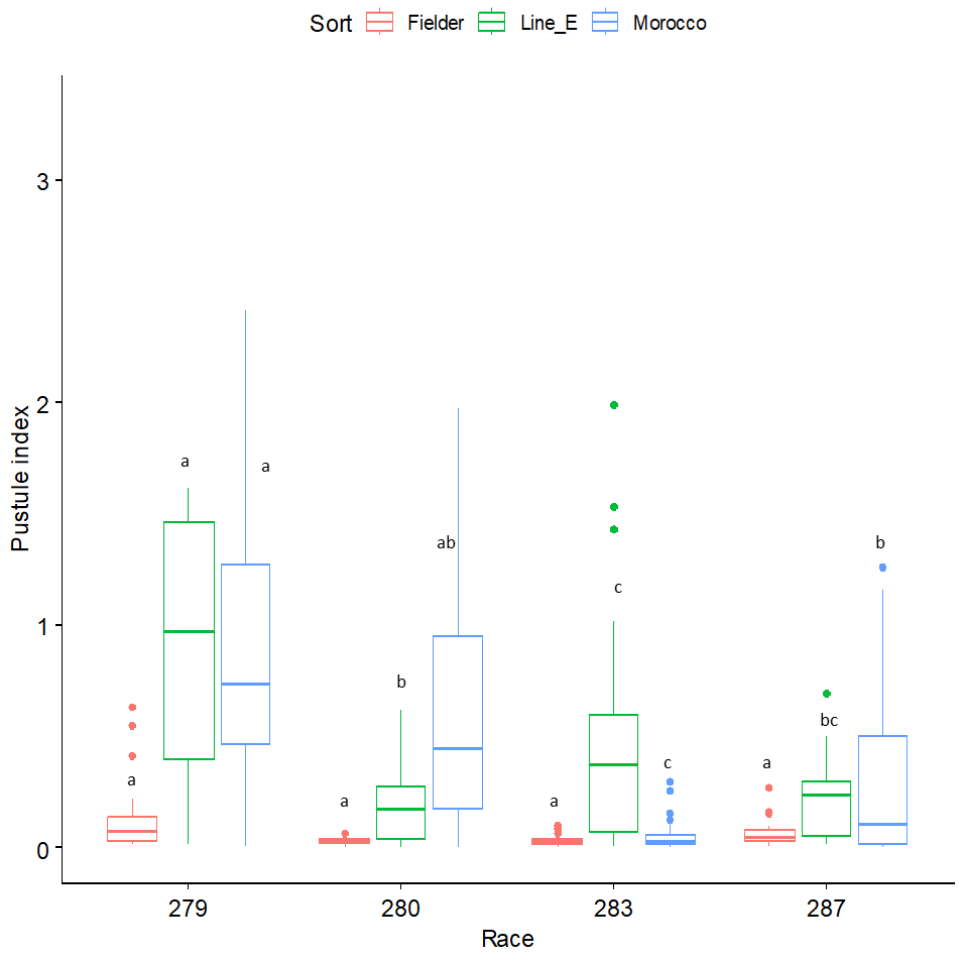

Figure 6. Pustule index for the four isolates grouped by wheat variety. Different letters indicate a significant ($p<0.05$) difference between the isolates within each variety group according to robust pairwise post-hoc test. The pustule index is calculated as a ratio of pixels from pustules to chlorotic and health pixels. P-value for wheat variety Fielder:0.104. P-value for wheat variety Line-E: 0.007. P-value for wheat variety Morocco: 0.019, (Table 5).

The chlorosis index when separated into the three wheat varieties showed significant differences between the isolates infecting the susceptible wheat variety Morocco and the resistant Fielder as seen in table 6 indicated by the p-value. Isolates infecting the susceptible Line E showed no significant differences between the means (Table 6; Figure7).

Table 6. Chlorosis index for the different isolates inoculated on the varieties Fielder, Morocco and Line E. Different letters indicate a significant difference ($p<0.05$) between the isolates according to robust pairwise post-hoc test

Isolate	**p-value***	**Robust pairwise post-hoc**
Fielder		
279		a
280	0.0132	b
283		b
287		b
Morocco		
279		a
280	0.0444	a
283		b
287		b
Line E		
279	0.251	a
280		a
283		a
287		a

**p-value represents the significant differences between the isolates grouped by the three wheat varieties.*

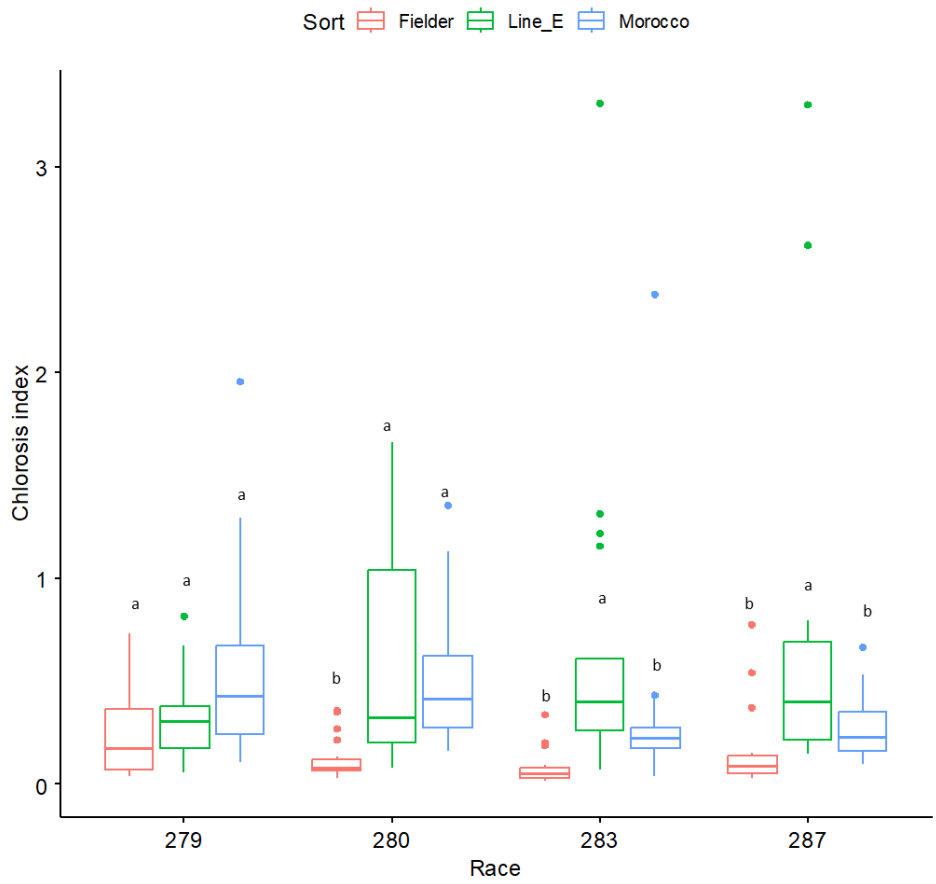

Figure 7. Chlorosis index for the four isolates grouped by wheat variety. Different letters indicate a significant ($p<0.05$) difference between the isolates within each variety group according to robust post-hoc test. The chlorosis index is calculated as a ratio of pixels from chlorosis to pustule and health pixels. P-value for wheat variety Fielder:0.0132. P-value for wheat variety Line-E: 0.251. P-value for wheat variety Morocco: 0.0444, (Table 6).

3.2 Experiment #2

The plants were scored after 10, 12, 14 and 16 DAIs. In the early stages of disease development at 10 and 12 DAIs the differences between the isolates were more pronounced. At 10 DAIs isolate 279 were significantly different from the other isolates with a higher disease score. Isolates 283 and 287 were also significantly different from each other with 283 scoring higher than 287 (Figure 8). At 12 DAIs isolates 279 and 283 were significantly differentiated from isolates 280 and 287 with a higher disease score (Figure 8).

At 14 and 16 DAI the isolates were closer in disease score with only 279 being significantly differentiated from 280 and 287 with a higher score (Figure 8). At 16 DAIs there were no significant differences between the isolates (Figure 8). The disease score across the three wheat varieties were lower than the first replicate due to the susceptible Line E being replaced with the semi-resistant variety Bob White.

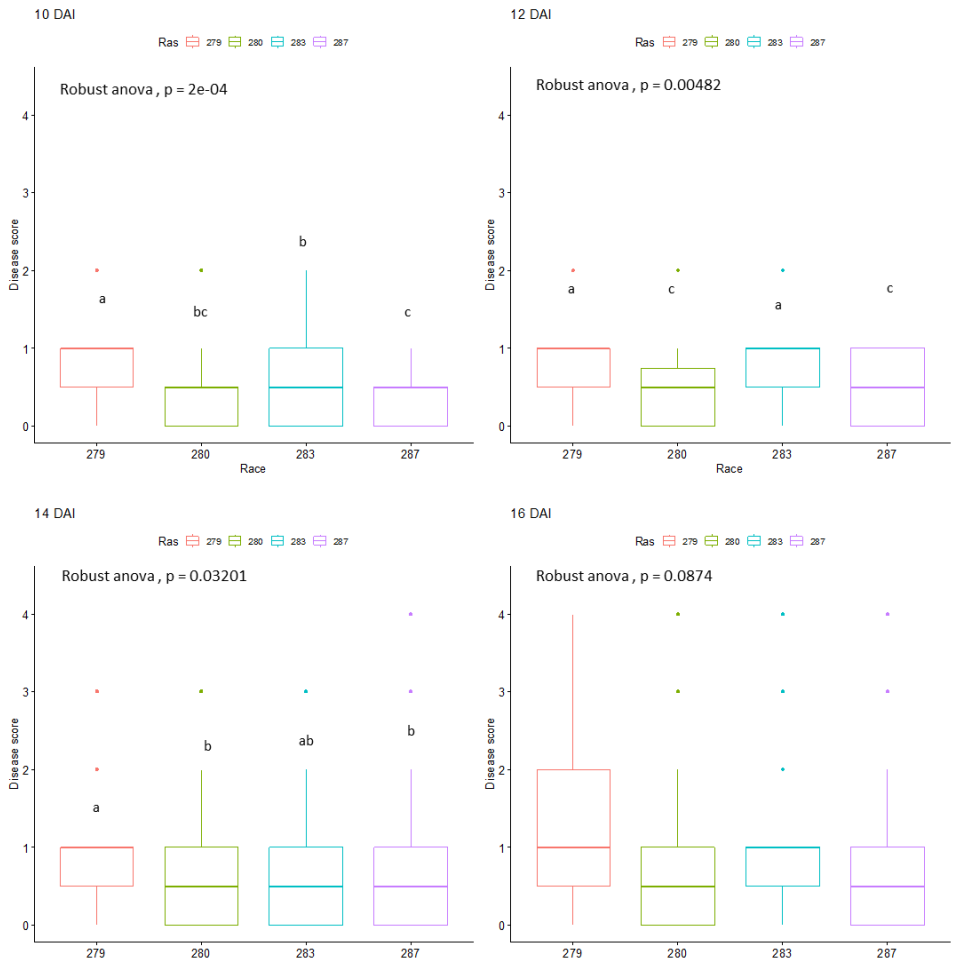

Figure 8. Disease score for each isolate on the three wheat varieties Morocco, Fielder and Bob white after 10, 12, 14 and 16 DAIs. Robust Anova indicates a significant difference at $p<0.05$. Different letters indicate a significant difference ($p<0.05$) from robust post-hoc.

Pustule index for the isolates in the experiments second replicate had no significant differences between the isolates on the different wheat varieties (Table 7; Figure 9). However there is a tendency for the isolates on the susceptible variety Morocco to developed high pustule indexes as compared to the resistant varieties Fielder and Bob white (Figure 9).

Table 7. Pustule index for the different isolates inoculated on the varieties Fielder, Morocco and Bob White. There was no significant difference ($p<0.05$) between the isolates according to robust post-hoc test.

Isolate	p-value*	Robust post-hoc
Fielder		
279		a
280	0.5516	a
283		a
287		a
Morocco		
279		a
280	0.8188	a
283		a
287		a
Bob White		
279	0.2004	a
280		a
283		a
287		a

**p-value represents the significant differences between the isolates grouped by the three wheat varieties*

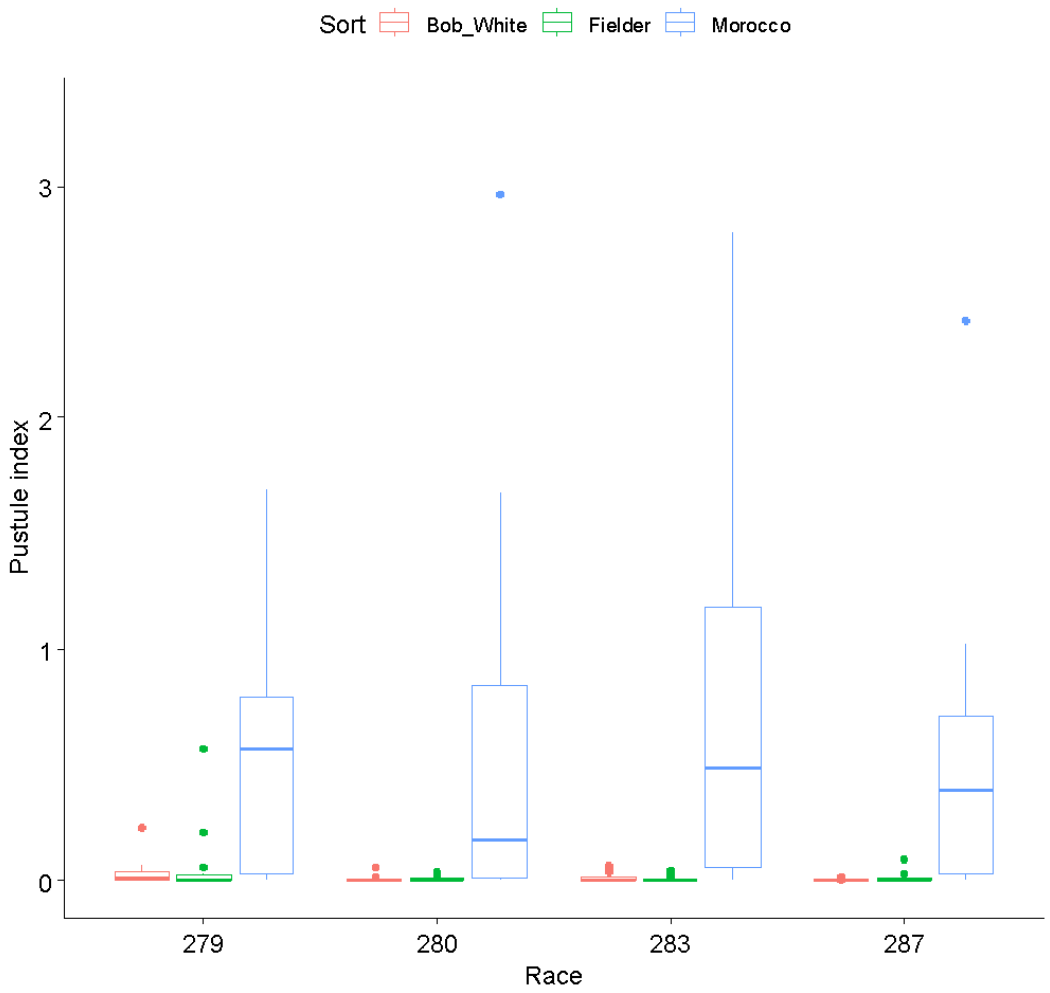

Figure 9. Pustule index for the different isolates inoculated on the varieties Fielder, Morocco and Bob white. P-value for wheat variety Fielder:0.5516. P-value for wheat variety Bob white: 0.2004. P-value for wheat variety Morocco: 0.8188, (Table 7).

Chlorosis index for the isolates in the experiments second replicate had no significant differences between the isolates on the different wheat varieties (Table 8; Figure 10). However there is a tendency for the isolates on the susceptible variety Morocco to developed high pustule indexes as compared to the resistant varieties Fielder and Bob white (Figure 10).

Table 8. Chlorosis index for the different isolates inoculated on the varieties Fielder, Morocco and Bob white. There was no significant difference ($p<0.05$) between the isolates according to robust post-hoc test.

Isolate	p-value*	Robust post-hoc
Fielder		
279		a
280	0.5862	a
283		a
287		a
Morocco		
279		a
280	0.2218	a
283		a
287		a
Bob White		
279	0.2454	a
280		a
283		a
287		a

**p-value represents the significant differences between the isolates grouped by the three wheat varieties*

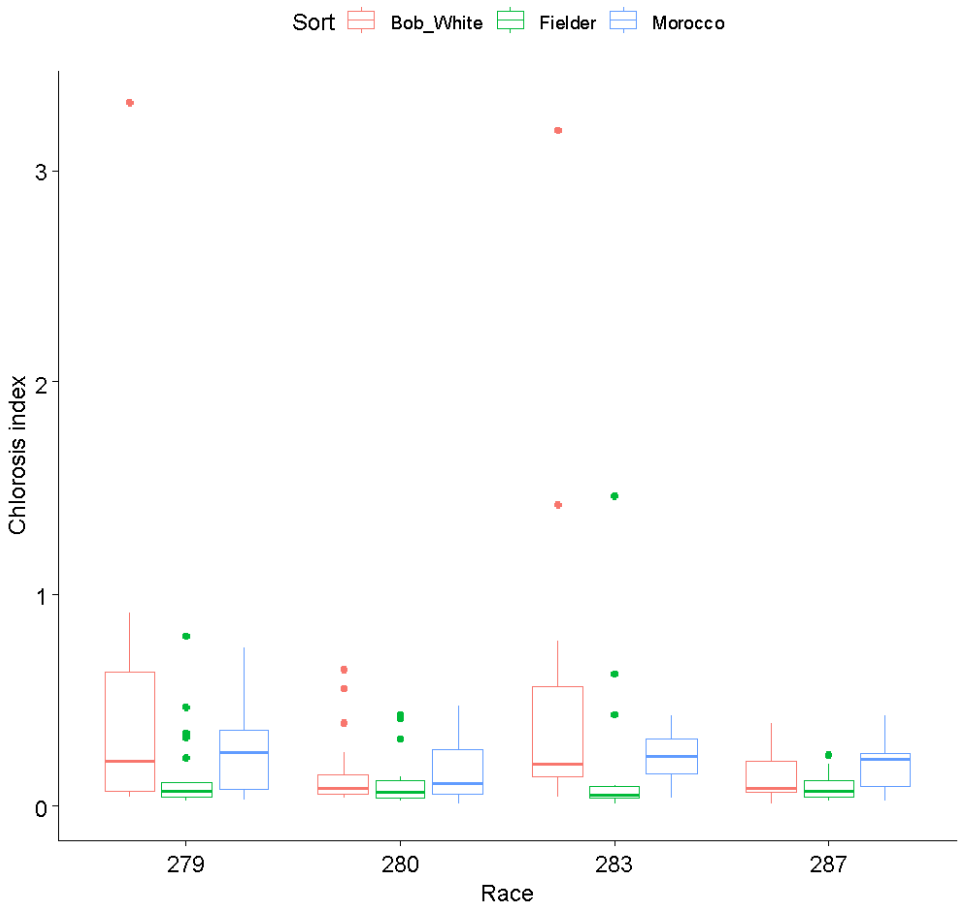

Figure 10. Chlorosis index for each isolate on the three wheat varieties. P-value for wheat variety Fielder:0.5862. P-value for wheat variety Bob white: 0.2454. P-value for wheat variety Morocco: 0.2218, (Table 8).

3.3 Combined data

Two wheat varieties were used in both experiments, the susceptible variety Morocco and the resistant variety Fielder. The overall disease score for the least complex race 279 were higher than the other more complex races, both on the susceptible Morocco and the resistant Fielder (Table 9).

When looking at Fielder, the isolates were not significantly different from each other in terms of pustule index which were low for all isolates (Figure 11). For the chlorosis index, isolate 283 developed significantly less chlorosis than the other isolates (Table 9; Figure 11). For Morocco, isolate 279 developed significantly higher pustule index than the more complex isolates 283 and 287 (Table 9; Figure 12). For the chlorosis index there were no significant differences between the means according to robust ANOVA (Figure 12).

Table 9. The overall disease score was calculated as the sum of all repeats for each isolate and divided with 42 (the number of repeats).

Variety	Isolate	p-value	Robust pairwise post-hoc	Overall disease score
Morocco	279		a	2.6
	280	0.0092**	ab	2.4
	283		b	1.5
	287		ab	2
Fielder	279		a	1
	280	0.0162*	ab	0.7
	283		c	0.4
	287		ab	0.6

**The p-value and post-hoc for Fielder is only for the chlorosis index. **The p-value and post-hoc for Morocco is only for the pustule index.*

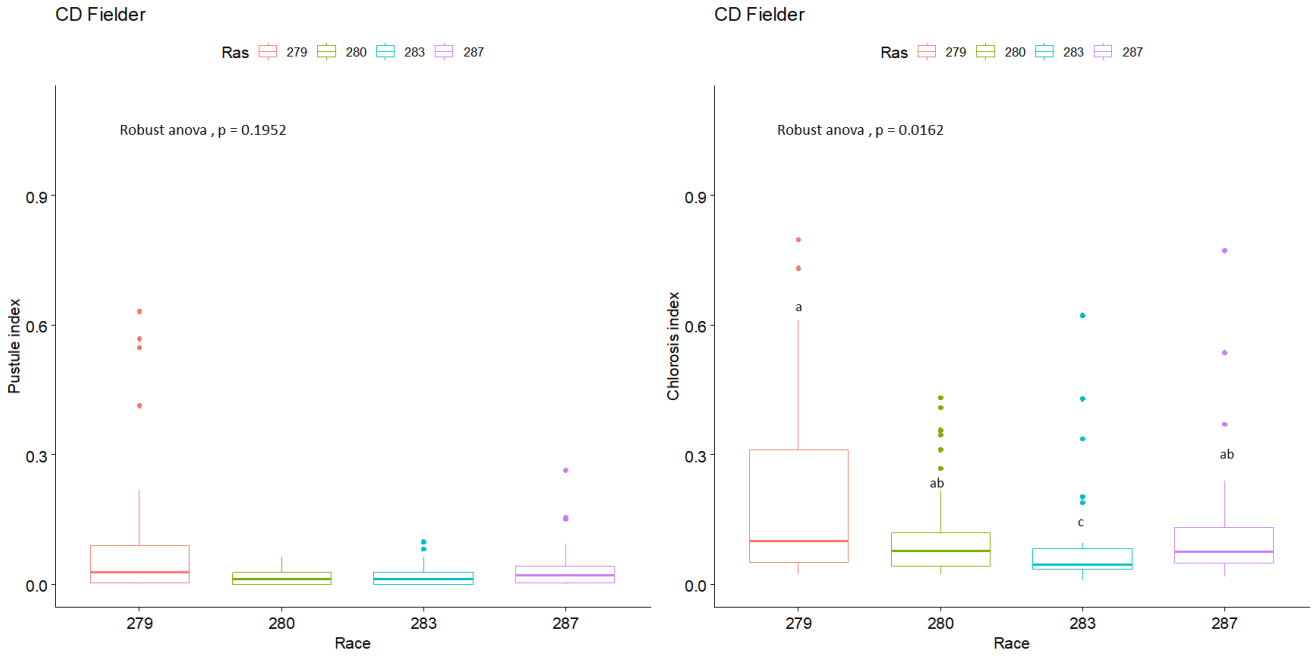

Figure 11. Pustule and chlorosis index for the resistant variety Fielder. Robust ANOVA indicates a significant difference between the means at p<0.05. Different letters indicate a significant (p<0.05) difference between the isolates according to robust pairwise post-hoc test.

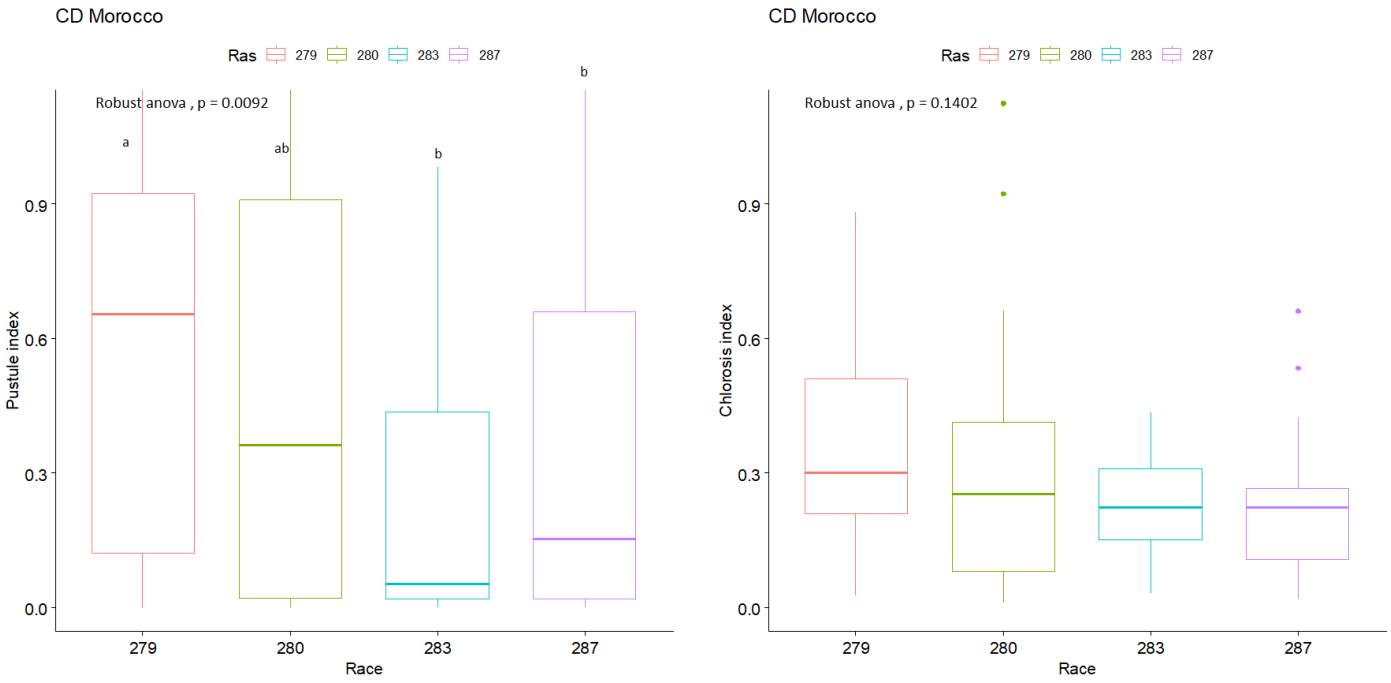

Figure 12. Pustule and chlorosis index for the susceptible variety Morocco. Robust ANOVA indicates a significant difference between the means at $p<0.05$. Different letters indicate a significant ($p<0.05$) difference between the isolates according to robust pairwise post-hoc test.

4. Discussion

The goal of this work was to investigate the effect of co-infections by different races of stem rust disease on wheat to evaluate the aggressiveness of the fungal pathogen *Puccinia graminis* when inoculated as a single race and in combination with other races. To allow for this, an image-based phenotyping method for stem rust disease assessment was developed and tested.

The result from the experiments shows that the more complex races inhibit each other when inoculating with a high spore high concentration, displayed as a several days longer days longer latency period and a slower disease development. With lower inoculation concentrations, the latency was close to equal between the different isolates in the second experiment. On the susceptible wheat varieties, the least complex Pgt race 279 and the intermediate 280 developed more severe disease than the more complex races 283 and 287.

In the beginning of this experiment the different races were labelled as weak and aggressive, with the less complex races 279 and 280 considered weak and the more complex races 283 and 287 considered aggressive due to the difference in the number of virulence genes between them. Our original hypothesis was that the "weak" races would develop as severe disease symptoms as the "aggressive" races when co-inoculated with each other. This experiment shows that some isolates, complex or simple, develop more disease symptoms when interacting with another isolate.

The method of attaching single leaves from wheat plants to a platform in order to inoculate and measure disease severity in a fixed area of leaf worked as intended. This method provides a base to build and develop further for research of pathogen interactions and for screening plant phenotypes in plant breeding programs.

4.1 Pustule and chlorotic symptoms

Of the four races, race 279 were considered simple in its virulence type as it carried a single known virulence gene. The intermediate races 280 carried three and race 283 four virulence genes and were only differentiated by one virulence gene.

Race 287 were considered complex as it carried seven identified virulence genes (Table 1). These are however only known virulence genes that have been tested for, the less complex races could contain other virulence genes that could make them more effective at infecting the hosts than the more complex races used in this experiment.

The results show that the more complex races develop disease symptoms later than the more simple races (Table 3; Figure 6) and that the pustule index was lower than for the more complex races (Figure 3; Figure 11). The overall disease score for the simple races compared to the complex races were generally higher than one or both of the complex races (Table 3), race 279 was always scored equal or higher than the more complex races on every wheat variety tested, while races 280 varied between a higher or lower score than one or the other of the more complex races. These differences were more apparent in the first replicate which had two susceptible wheat varieties and one resistant variety while the second replicate had two resistant varieties and one susceptible wheat variety.

4.2 Virulence and host resistance genes

All four races share five virulence genes *Sr5, Sr8a, Sr9a, Sr9g, Sr17* and *SrMcN*. In addition to these tested Pgt races have one or more virulence genes up to 7 extra for race 287 (Tabel 1).

It is suggested that there is a fitness cost for pathogens to carry multiple virulence genes . Bahri et al. (2009) carried out a competition experiment of 16 isolates of *Puccinia striiformis* f.sp *tritici* whose genotypes differed by a single virulence gene not matched by the host resistance genes. They concluded that the isolates with the extra virulence gene that was not needed for infection gave them a selective disadvantage (Bahri et al. 2009). This supports our results, as race 279 carrying fewer virulence genes is able to develop disease symptoms earlier and more severe than the more complex races. Race 279 has no additional virulence gene, and proved more effective at infecting the wheat varieties, even the resistant Fielder. This could suggest that a more complex race has some cost for having more virulence genes.

4.3 Data and statistics

When the data was found to not be normally distributed, both visually and through tests, non-parametric test had to be used. After this evaluation non-parametric tests

of robust ANOVA and pairwise post-hoc were chosen to be used rather than of ANOVA and Tukeys HSD tests.

For the image analysis the leaf area was assumed to be the same for every wheat variety. However, due to different phenotypic traits in the wheat varieties, especially for Line E which had notably thinner leaves than Morocco, Fielder and Bob white, the disease area by the PlantCV script calculated might not be larger than those of Morocco or Fielder.

4.4 Source of errors

Another difference between the replicates that might have played a key role to the difference in disease area between the races is the spore concentration used. The first experiment wheat plants were inoculated with a mixture of 0.5 mg of spores suspended in 1 ml of Tween-water. This high concentration may have inhibited the growth of mainly the complex races as there is a several days difference in latency period between the replicates, since all other conditions were the same.

During the experiment in the growth chambers the plantlets of the wheat varieties Line E and partly of variety Morocco suffered from water stress. It was found that it was due to a tilt in either the tray that held the 16 pots for each variety or the growth chamber itself. The tilt made the water only reach the front half of the trays if not liberally watered. This resulted in shrivelled leaves and chlorosis on several plants of Line E and some plants of Morocco losing turgor pressure.

5. Conclusions

In the work presented here, an image-based phenotyping method to assess the stem rust disease development on wheat seedlings was developed. The results from the two co-inoculation experiments show that less complex races are more aggressive when infecting a wheat variety. This indicated that sexual populations of stem rust may pose just as large threat to wheat production as aggressive clonal linages. This information should be considered when developing breeding strategies and other disease management methods.

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Acknowledgements

Thank you to my supervisor Anna Berlin and examiner Björn Andersson. And a special thank you to Miguel Ángel Corrales Gutiérrez for providing the wheat varieties and for your assistance in developing image analysis method and for your expertise in statistics.

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