Fungicide tolerance in *Phytophthora infestans*

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Swedish University of Agricultural Sciences, SLU
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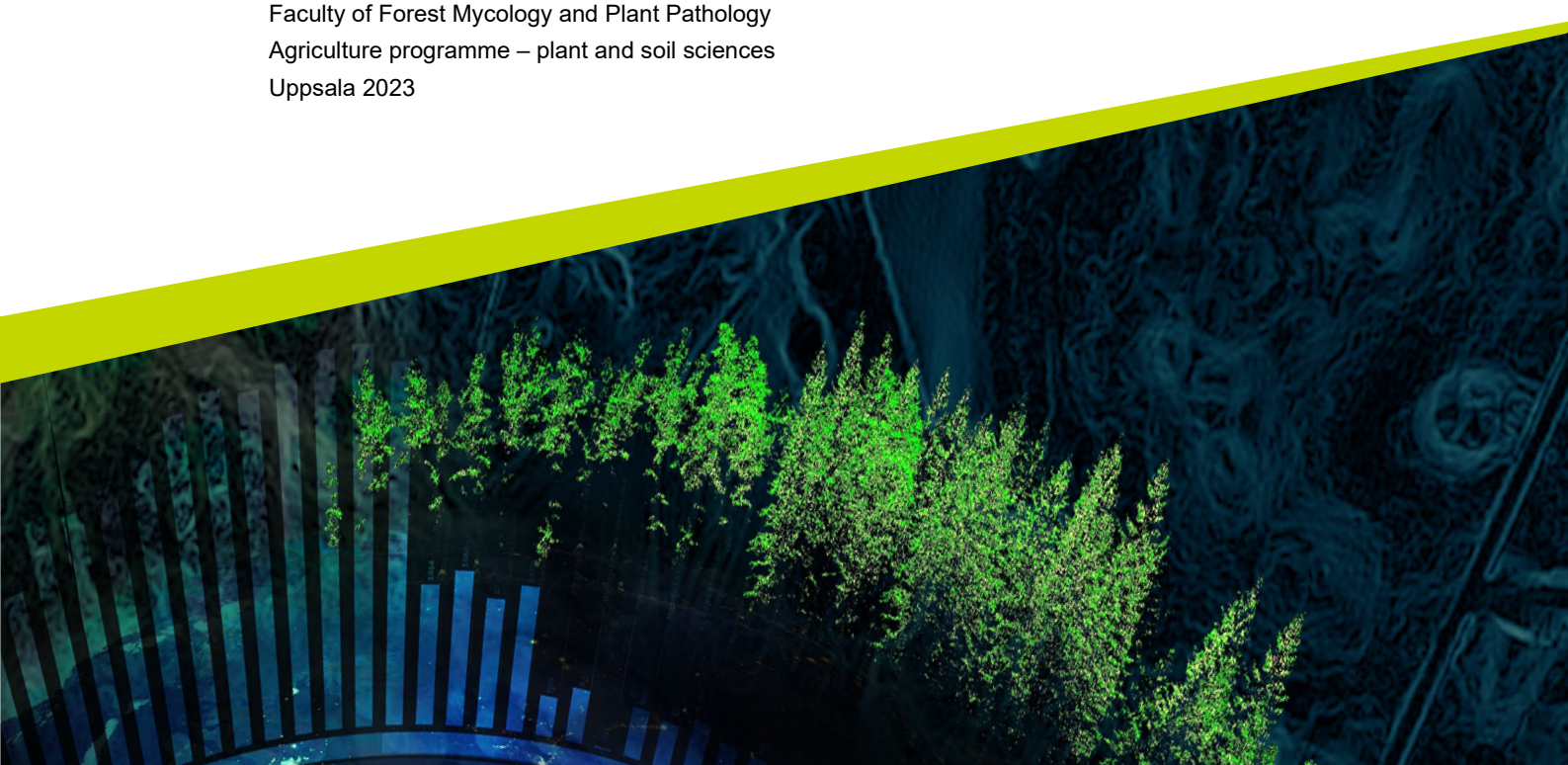

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Abstract

For the last 180 years, the pathogen *P infestans*, responsible for the late blight disease, has been a severe problem for farmers. Despite the large efforts and financial investments done in the management of the disease, 15-30% of the potato production is lost to this disease globally each year. The late blight requires a high level of control, where fungicides play a crucial part, to protect the quantity and the quality of the harvest. The use of fungicides is associated with unfavourable consequences, where the risk of a reduced sensitivity to fungicides in the pathogen is one of the more severe. In this study, the aim was to examine how the use of different dosages of the fungicide Infinito affects the genotypic and phenotypic makeup of a *P. infestans* field population. The population was genetically characterized by using a 12-plex Simple Sequence Repeats (SSRs) method. Sensitivity to the fungicide *in vitro* was estimated based on radial growth on Infinito-amended rye-pea agar. The result of the study revealed a population with an unexpectedly narrow genotypic diversity. In the *in vitro* fungicide tests, no distinction between isolates from treated and untreated plots were observed. Neither were there any signs of selection towards more tolerant genotypes and no significant differences between individual isolates was observed.

Keywords: Phytophthora infestans, Fungicide tolerance, Fungicide dose

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1. Background

1.1. Background to study

Fungicides play a crucial role in the management of potato late blight, caused by *Phytophthora infestans*. The extensive use of fungicides leads to a risk of fungicide resistance development within *P. infestans* populations, which would diminish the efficacy of fungicides, and as a result, the possibilities to control the disease.

In the summer of 2020, indications of resistance to the fungicide Infinito (propamocarb 625 g/l, fluopicolide 62,5 g/l) were reported from a field trial located in Southern Sweden. The purpose of the trial was to examine the effect of different control strategies where three different dosages of the fungicide was tested: quarter, half, and full recommended dose.

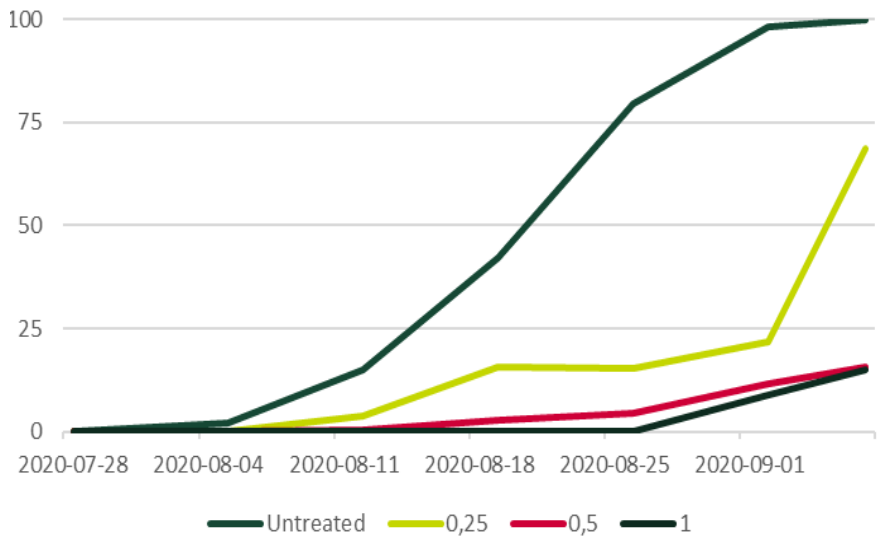

*Figure 1. Development of late blight (*P. infestans*) in a field trial with treatments with different dosages of Infinito during the growing season 2020. Dose-response curve from untreated plots (untreated) and treatments with 25% (0.25), 50% (0.5), and 100% (1) of the recommended dose.*

The dose-response curve from the experiment (Fig. 1) displays a reduced effect of the lowest dose of fungicide (quarter of the recommended dose) in early August. Infections could also be observed in plots treated with half and full fungicide dose from late August.

1.1.1. Objectives

Fungicide application exerts selection pressure on fungal populations, promoting the survival and dispersal of individuals expressing a higher fungicide tolerance. If the proportion of tolerant "individuals" in the population becomes large enough, this may result in a reduced treatment effect under field conditions.

The aim of this study was to examine how the use of different fungicide dosages used to control potato late blight affects the genotypic and phenotypic makeup of a *P. infestans* field population. The objectives were to genetically characterize the *P. infestans* population by using molecular markers (microsatellites, SSR - Simple Sequence Repeats) to determine the population structure. Fungicide tolerance determined *in vitro* was used to phenotypically characterize the pathogen population. The study was done to examine whether there were any indications of selection from the use of varying fungicide dosages during a single growing season in the studied field population of *P. infestans*.

Hypothesis:

- Due to selection pressure exerted by fungicides, *P. infestans* isolates collected from treated plots have a lower sensitivity to fungicides compared to isolates from untreated plots.
- Isolates collected from plots treated with a higher dose of fungicide will show a higher tolerance to fungicides than isolates collected from plots treated with lower dose.
- Selection caused by fungicide use will cause a change in the genotypic population structure.

1.2. The host – Potato (*Solanum tuberosum*)

Potato (*Solanum tuberosum*) is a tuber crop, originating from the Andes mountains of South America, where more than 4000 wild varieties can be found (CIP 2021). It is the most important non-cereal crop globally (Birch et al. 2012), and in 2017 the total global production exceeded 388 million tonnes (FAOSTAT 2019). In 2020, the top two producers were China and India, where almost a third of the global potato yield was produced (FAO).

Through its high nutritional value, high yields, and low production costs, the potato plays a vital role in the food security in many developing countries (CIP 2021). The crop is favoured by cooler temperatures (optimum 18-20°C) but has a high adaptability to different climates, from temperate to tropical, enabling it to be cultivated globally (FAO). Although being a perennial plant, it is cultivated as an annual crop, planted, and harvested every year. The crop is clonally propagated leading to a narrow genetic base.

The crop was introduced in Europe 1530 (Birch et al. 2012). In Sweden, potato growing had a very slow start after its introduction in 1658. Despite being known to have edible bulbs it was planted as an ornamental plant and not grown at a larger scale until Sweden suffered a crop failure crisis in the late 1700s. However, it did not become a staple crop in Sweden until the early 18th century (Bodensten 2021). In 2020, around 24000 hectares were used for growing the crop, with a yield of 878700 tonnes (SCB 2019; Jordbruksverket).

1.3. The late blight disease

For the last 180 years, the pathogen *P infestans*, has been a severe problem for farmers. It is responsible for the late blight disease, by far the most important and devastating disease in potato production, especially in areas with a cool, humid climate (Agrios 2005; Birch et al. 2012). The first cases of late blight disease were reported around the docks of New York City and Philadelphia in 1843. The disease spread rapidly to neighbouring states, leaving blighted potato fields with losses up to 50% in its wake (Stevens 1933). In June 1845, the disease reached Europe, where it was first discovered in Belgium. Three months later it reached Ireland, where the extremely devastating potential of this disease was demonstrated (Bourke 1964). The rural poor population relied on potato as a staple crop and reoccurring epidemics during the 1840's, which left field totally demolished and the harvest inedible from tuber blight, resulted in mass-starvation, a crisis referred to as 'the Irish famine' (Zadoks 2008). Goodwin et al (1994) accredit the global dispersion of the pathogen to international seed trade.

Today, the disease can be found everywhere potato is grown. Despite the large efforts and financial investments done in the management of the disease, 15-30% of the potato production is lost to this disease globally each year. The annual cost for control and crop loss in the European Union is estimated to more than €1 billion (Haverkort et al. 2008; Haverkort et al. 2009).

1.4. Symptoms of the disease

The late blight disease is identified by water-soaked, dark lesions on both leaves and stems. The lesions enlarge rapidly during suitable conditions, leaving the whole leaf necrotic. If the weather is humid, sporangia and sporangiophores are produced on infected tissue, and the resulting sporulation can be seen at the edges of the lesions as a halo of white, mildew-looking growth, mainly on the abaxial surface of the leaf. As the initial lesion is spreading, the whole plant soon becomes necrotic, and in favourable weather conditions entire fields may die within a week if not treated (Agrios 2005).

If sporangia are washed from lesions on the foliage to the soil the tubers may become infected and result in tuber blight. Tuber infections are caused by zoospores produces by the sporangia. The zoospores move in the soil water to reach the tubers. The symptoms are dry, brown to purple patches which can penetrate deep into the flesh (Agrios 2005). The rot continues to develop after harvest and infected tubers are prone to secondary infections and tend to rot during storage (Medina et al. 2011).

1.5. Control of the late blight disease

Preventing measures to limit inoculum sources and potential infection is an important part in managing plant diseases. To control late blight, certified disease-free seed of tolerant cultivars should be used. The pathogen is favoured by a humid, cool climate and fields where the crop is grown should be well drained, and preferably open without any shading vegetation. Some Solanaceae weeds are hosts for the pathogen, and these, as well as volunteer potatoes, should be considered as a source of inoculum both prior to planting and throughout the season (Andersson et al. 2003; Grönberg et al. 2012).

1.6. Fungicides and fungicide resistance

1.6.1. Fungicides

One of the first fungicide was the so-called Bordeaux mixture, based on lime and copper compounds. Between 1940 and 1960, fungicides developed were primarily contact fungicides (Hawkins & Fraaije 2018).
Contact fungicides works by covering the leaf surface and prohibiting infection. They often have a broad-spectrum activity, targeting a wide range of fungal diseases, and several modes of action (MOA) where each MOA targets a specific

enzymatic process of the pathogen. Contact fungicides require recurring treatment since growth of foliage surface disrupts the protective layer (Wyenandt 2020).
In 1970s, systemic fungicides were introduced (Hawkins & Fraaije 2018). Systemic fungicides penetrate the cuticle of leaf and stems and are redistributed in the plant. Mostly, they have a specific mode of action, targeting a single process, and a narrower spectrum of effectivity. Compared to contact fungicides, they provide control for a longer period and require a lower application rate.
Translaminar fungicides have properties from both mentioned groups; they are absorbed by the leaf tissue where applied, protecting both sides of the leaf (FRAC 2019).

Potato late blight requires a high level of control to protect the quantity and the quality of the harvest. Regular sprayings with a mix of contact, translaminar and systemic fungicides, is standard practice. Normal interval for treatment is seven days, with even shorter intervals, 4-5 days if the weather is very favourable for the pathogen or the growing of the crop is intense. The interval may increase to 10-14 days if weather is unsuitable for the pathogen (Jordbruksverket). Although the risk of serious infection may be reduced by using fungicides, the use is associated with unfavourable consequences. 10-20% of the total production cost for potato in EU and US goes to chemicals for controlling the disease (Haverkort et al. 2009). The use of fungicides also poses a threat to environment and non-target organisms and may lead to reduced sensitivity to fungicides in the pathogen (FRAC 2019).

1.6.2. Fungicide resistance

Fungicide resistance is an acquired, heritable reduction in sensitivity of a fungus to a specific fungicide (FRAC). Van den Bosch et al. (2011) describes the development of fungicide resistance through three key phases, where resistant strains arise through mutations. The outcome of mutations is in many cases neutral, or even unfavourable, to the organism. However, if the mutation occurs in genes encoding target sites of fungicide, a single mutation may lead to full resistance. In other cases, the shift within the population is more continuous, as numerous mutations increases. In the next phase, which Van den Bosch et al (2011) calls the ‘selection phase’, the fraction of the pathogen population, carrying the resistance, increases. Each time fungicides are applied a selective pressure is exerted on the pathogen population where genotypes with a reduced sensitivity to the fungicides will survive and reproduce while individuals with sensitive genotypes die. In the final phase, the resistance has advanced to levels that affects the control efficacy of the fungicide, and the control method may have to be altered to maintain effective.

In general, pathogen populations with a high genetic variation tend to have a greater potential to adapt to changing environments and hence have the potential to develop

fungicide resistance (McDonald & Linde 2002) more quickly. Generally, pathogens are considered as high-risk to develop resistance if the life cycle includes many and short disease cycles/season, sexual reproduction, and if resistance to new products has evolved rapidly (FRAC 2019).

Phytophthora infestans expresses several risk-factors for the development of fungicide resistance; a polycyclic life cycle, the reproduction of a high number of spores, a combination of sexual and clonal reproduction, a large genome and a high adaptability as shown by its potential to overcome host resistance. Yet, it is only considered a medium risk pathogen by FRAC (2019). This is since the pathogen developed resistance quickly to phenylamide fungicides, but not at all to fungicides within the CAA-, QoI-, QiI-, cymoxanil-, carbamates-, and organotins groups (FRAC 2019). However, recent epidemics of the disease are connected to the emergence of more aggressive lineages, for example the lineage EU_37, which has a reduced sensitivity to fluazinam (EuroBlight. 2019).

The management to reduce resistance towards fungicides is to alter products used or use it in a mixture with other substances with different MOA. Other ways that are often recommended are to restrict the number of applications and to maintain the manufacturers recommended dose (FRAC). However, reduced fungicide doses have been used, mainly in areas with a low infection pressure, as a mean of reducing monetary expenses. While some studies have implied that reduced dose enhance development of resistance (FRAC), others claim the opposite. Van der Bosch et al (2011) reviewed several studies of dose/resistance and found that a higher dose reduces the time for resistance to evolve by killing off sensitive individuals, leading to less competition of resources for the tolerant ones.

1.7. The causal agent; *Phytophthora infestans*

1.7.1. Biology of the pathogen

When discovered, the pathogen was named *Botrytis infestans* but the name was changed in 1876 by Anton de Bary, giving rise to the genus *Phytophthora,* Greek for “plant destroyer” (Lamour 2013). Much evidence suggests the central highlands of Mexico as the probable geographical origin of the pathogen (Fry 2008; Grünwald & Flier 2005). Specific resistance genes in the plant genome were first discovered in two *Solanum* species, endemic to Mexico, indicating co-evolution between the pathogen and its host plant. *P. infestans* has two mating types, and both can be found in a 1:1 ratio in Mexico (Flier et al. 2003; Grünwald et al. 2001).

The pathogen shares many morphologically similarities with true fungi, i.e filamentous growth, but is more closely related to algae and belongs to the phylum Oomycota in the Stramenopila family (Lamour 2013). Some major distinctions between the oomycote and true fungi are the composition of the cell wall, which primarily consists of cellulose in oomycetes while it consists of chitin in true fungi, the presence of motile zoospores with whiplash- and tinsel type flagella and vegetative growth with diploid cells (Fry & Goodwin 1997).

The genome of *P. infestans* was fully sequenced in 2009 and is remarkably large and complex. It consists of 240 Mbp, where approximately 74% of the genome is repetitive DNA displaying a very high flexibility. (Haas et al., 2009),

1.7.2. Population structure

In Europe, *P. infestans* populations were generally considered to belong to a single clonal lineage of mating type A1 until the mid-1970s (Goodwin et al. 1994). In 1984, a new *P. infestans* population distinguished by a much greater genetic diversity, earlier unknown allozyme genotypes and the A2 mating type was found in several European countries including Sweden (Hohl & Iselin 1984; Kadir & Umaerus 1987; Fry et al. 1993). Findings of this new population was soon reported from countries in Asia and Africa (Drenth et al. 1993).

Although both mating types are now widely distributed globally, the overall population of *P. infestans* is still characterized by asexual reproduction. In Europe, 70% of the samples belongs to a few clonal lineages. The frequency of genotypes in European populations may alter quickly making the adaptation of control measures, such as choice of fungicides and/or cultivar, and the rates of spraying, more complex (Euroblight 2021). However, compared to most of the world, the population structure of *P. infestans* differs in Sweden and the Nordic countries in much the same way as in Toluca Valley, central Mexico. These populations are characterized by short-lived, genetically diverse isolates with unique SSR genotypes (Cooke et al. 2019). These unique SSR genotypes probably originates from oospore inoculum. This assumption is supported by an even mating type distribution and the formation of oospores in Swedish potato fields (Dahlberg et al. 2002).

1.7.3. The asexual life cycle of *P. infestans*

The asexual life cycle is initiated when overwintering potato tubers, infected with mycelium of *P. infestans*, give rise to a diseased seedling (Agrios 2005). Under suitable weather conditions, characterised by high humidity and cool temperatures, sporangiophores develops through the leaf's stomata. Each sporangiophore may

produce several lemon-shaped, airborne sporangia. When mature and during suitable climate conditions, the sporangia are disjoined from the sporangiophore and carried to surrounding plants, primarily by air, which facilitates a fast dispersion of the pathogen (Fry 2008).

If the sporangia land on a susceptible host in humid conditions with temperatures between 20-25 degrees, the sporangia germinate directly. When temperatures are lower, between 10-15 degrees, 3-8 motile zoospores are released. These are motile in water and can swim towards an appropriate entry point, for example stomata. These zoospore are short lived, but may encyst to extend their survival and infection potential (Fry 2008).

The asexual cycle is of a polycyclic nature and the period from infection to sporangia formation only takes a few days, leading to a considerable number of asexual generations and new infections during each growing season (Drenth et al. 1993; Fry, 2008). The asexual form of the pathogen can only survive between seasons in living host tissue in tubers in cull piles, infected tubers left in the field during harvest, in infected seed or on other Solanaceous plants (Agrios 2005).

1.7.4. The sexual life cycle of *P. infestans*

As a heterothallic pathogen, the sexual reproduction requires the presence of both mating types, A1 and A2. Interaction between hyphae of opposite mating types generates the production of the sex organs antheridium and oogonium, each producing haploid nuclei. When these haploid nuclei are fused, a diploid oospore is produced. The oospore carries genetic material from both parents, resulting in a great population diversity (Brus-Szkalej 2019). Oospores increase the risk for early epidemics and a possibly more rapid disease development, and several studies implies that oospores can act as primary inoculum (Andersson et al. 1998; Widmark et al. 2007). Oospores differ from sporangia and zoospores by having a thick, robust cell wall making them able to survive for several years in the soil. A study by Medina & Platt (1999) showed that, even after being exposed to rough climate conditions, a big part of oospores was able to infect its host plant. In this way, the presence of oospores overwintering in the field also increases the amount of primary inoculum (Medina & Platt 1999).

The Swedish *P. infestans* population is characterized by high genetic diversity. In a study by Brurberg (2011), 169 out of 191 samples in Sweden had unique SSR genotypes. One theory as to why the population is primarily sexual in the Nordic countries is an adverse climate for clonal overwintering of mycelia favouring oospores as a survival strategy (Brurberg et al. 1999). When present in the soil, oospores can result in earlier outbreaks compared to regions with a population

dominated by clonal reproduction. For the clone to spread, spores first must be produced from a living source, such as a diseased tuber giving rise to an infected seedling. A study by Yuen et al (2012) indicates that the first *P. infestans* genotypes ("individuals") present in a field have the best chance of becoming dominating in the pathogen population.

2. Material and method

2.1. Sample collection

Samples of *P. infestans* were collected in a field trial located at Mosslunda gård, near Kristianstad, Sweden. The purpose of the trial was to determine the dose-effect curve of fungicides used for treatment of late blight. The field trial was set up in a randomized block design and the potato crop was treated with a fixed spraying program with different fungicide dosages (full dose, half dose and quarter of recommended dose) once a week. Control plots were not treated with any fungicides. The cultivar in the trial was Kuras which has a high to very high resistance to late blight disease (Europotato 2010).

Potato plant leaflets that showed characteristic symptoms of infection of *P. infestans* were collected from each plot of the trial. The collected leaflets were immediately placed individually in plastic zip-lock bags to avoid cross-contamination between samples. Preferably, leaflets with a single lesion and active sporulation were collected, but since the collection was late in season, leaflets with multiple lesions were also collected. The sampels were sent via post to SLU, Uppsala.

2.1.1. Population structure – Genotyping

The samples were split in half, one half was put on FTA-cards for the DNA sampling according to EuroBlight (2013). A visible lesion of every leaf was pressed with the sporulating side facing down on the sampling area of the FTA card to capture the pathogens DNA. Plant residues were removed, and the cards were allowed to air dry before they were packed and sent to the James Hutton Institute for genotyping. The genotyping was done by DNA fingerprinting using a 12-plex Simple Sequence Repeats (SSRs) method (Li et al. 2013).

2.1.2. Isolation of *Phytophthora infestans*

Rye-pea agar was prepared by letting 300 grams of whole rye soak over-night in distilled water. The following day, 400 grams of frozen green peas and water was added, and the mixture was boiled for 60 minutes. Rye and pea particulates were filtered from the solution using a fine strainer and the liquid was collected and diluted up to 5 L. Bottles with agar equivalent to 1,5 % of the rye-pea solution were autoclaved and left to cool down. A semi-automatic agar plate dispensing equipment was used to fill plates with 18 millilitres of the agar solution. The plates were stored at room temperature until used.

The second half of each sample was placed on a centimetre-thick slice of a freshly cut potato tuber (cv Asterix) in a Petri dish. The slice was incubated at room temperature to promote mycelial growth. Mycelium from the tubers were then transferred to plates with rye-pea agar. When the mycelium had grown out on the plate, two small pieces with visible hyphae of *P. infestans* were cut out with a sterilised scalpel and placed on a new pair of agar plates and incubated in +20 ºC. This procedure was repeated until other suspected contaminants were eliminated by attempting to transfer only the *P. infestans* and the samples were clean. The plates with the pure cultures were incubated at +12 ºC in darkness.

2.1.3. Phenotyping for fungicide insensitivity

Sensitivity to the fungicide *in vitro* was estimated based on radial growth on Infinito-amended rye-pea agar. The test was performed at three different concentrations of Infinito®: 0.1, 1.0 and 10 ml per litre agar. The concentrations were obtained from a previous experiment performed by Jönsson & Olsson (2015) and were chosen so that the growth of *P. infestans* was almost totally hampered on the highest concentration while it grew almost unimpeded on the lowest. A concentration on which the impact of the fungicide was significant, but still allowed the mycelia to grow, was chosen between the highest and lowest dose. 100 µl of the diluted fungicide-solution, were manually pipetted at the centre of a solidified rye-pea agar plate. An L-shaped, disposable spreader was used to dispense the solution evenly on the agar surface. To allow for the solution to diffuse evenly into the agar the plate was stored in room temperature for approximately 12 hours. Control plates contained non amended rye-pea agar. A 10-mm-diameter plug of actively growing mycelia was cut out from each isolate and placed at the centre of a fungicide-infused plate. The procedure was repeated four times for each isolate and concentration, resulting in a total of 16 agar-plates per isolate: 4 per concentration and 4 controls. After 20 days, the mycelial radial growth was measured on the fungicide-prepared plates relative to the mycelial growth on the control plates and was calculated as relative growth compared to control. On isolates where no mycelial growth was

visible to the naked eye, a dissection microscope was used to determine signs of growth.

2.1.4. Analysis and statistics

Several analyses were performed to investigate the population structure of the collected samples. Principal coordinate analysis (PCoA), based on the genetic distance was used to present dimensional depiction between individuals. A phylogenetic tree based on Bruvo genetic distance was used to represents the relationships in the population. Minimal Spanning Network (MSN) was used to analyse and visualize genetic relationships within and the population. The G/N ratio, where the number of unique multi locus genotypes (MLGs) was divided by the total number of samples, was used to calculate the genotypic diversity .

The Excel add-on GenAlEx (Peakall et al. 2012) was used to calculate the PCoA while PoppR (Kamvar et al. 2014) was used for calculating genetic distances (Bruvo et al. 2004) and constructing the MSN. All analyses of the phenotypic results were performed in JMP (JMP® SAS Institute Inc).

3. Results

3.1. Genotypic variation: SSR markers

A total of 65 isolates were sent for genotyping. The SSR genotyping revealed that a great majority, 56 of 65, of the sampled isolates were identified as belonging to the clonal lineage EU_41_A2 (Table 1). Three of the isolates had unique SSR multi locus genotypes (MLGs) and were categorized as 'others' and were a probable result of sexual recombination. Six of the isolates failed to be genotyped. The 'other' genotypes were evenly distributed and present in all except the plots treated with half the recommended fungicide dose.

The total number of genotypes found among the isolates were 6, giving a G/N ratio of 0,10. A result close to 1 indicates a high genotypic diversity, while a low indicates a narrow genotypic diversity. The low G/N ration indicating a population with a small genotypic diversity, which corresponds with the high number of the clone EU_41_A2.

Table 1. The distribution of genotypes in the different treatments.

Field treatment	EU_41_A2	Other	Failed	Total
Control	21	1	0	22
¼ dose	13	1	4	18
½ dose	12	0	1	13
Full dose	10	1	1	12
Total	56	3	6	65

3.1.1. Phylogenetic tree

The phylogenetic tree based on the genetic differences within the sampled field population showed that the EU_41_A2 samples separate into three clusters (Fig. 2). The length of the branches connecting the clusters indicate the genetic distance between them.

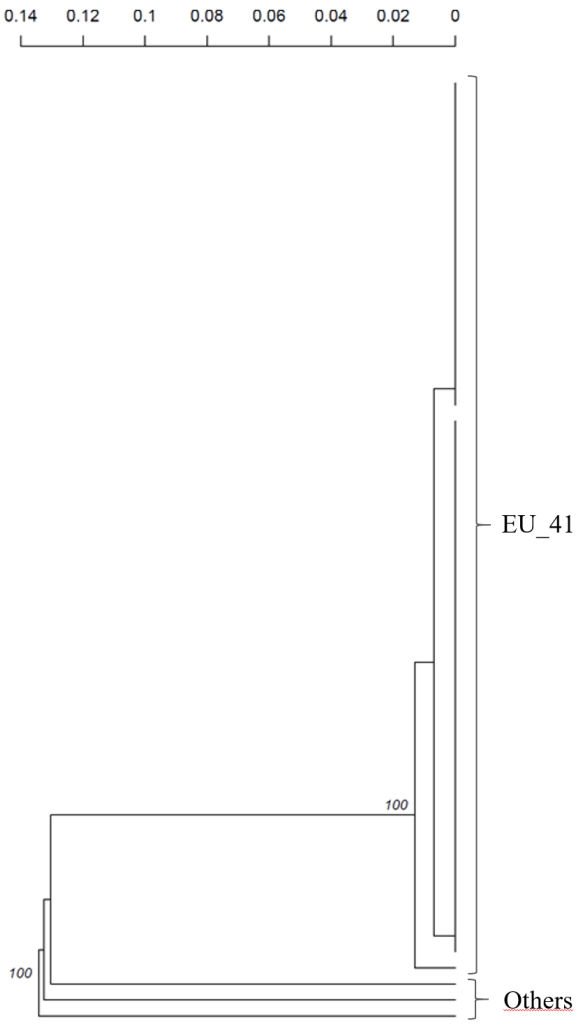

Figure 2. Phylogenetic tree based on Bruvo genetic distances of the sampled isolates.

3.1.2. Principal Coordinate Analysis

The PCoA reveals a population with a narrow genotypic diversity, which corresponds with the result from the phylogenetic tree. In the analysis, clades with a high relatedness are displayed as being tightly clustered. All the EU_41_A2 samples are tightly clustered, with two of them overlapping (Fig. 3).

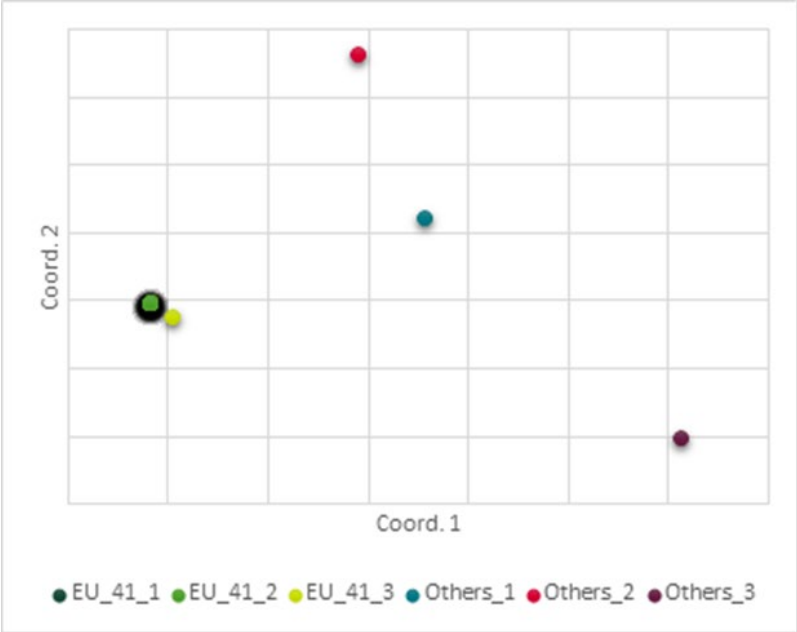

Figure 3. Principal Coordinate Analysis (PCoA) of the studied Phytophthora infestans *isolates, illustrating the genetic pattern within the population. The clades EU_41_1 and EU_41_2 is overlapping. Axis 1 and axis 2 respectively explained 55 and 27% of the variance.*

3.1.3. Minimum Spanning Network

The lines in the MSN (Fig. 4) represent minimum genetic distance between samples with the thickness of the branch indicating the level of relatedness between the individuals. More closely related “circles” are connected by a thicker line, while more distantly related samples have lighter and thinner lines connecting them. As in the previous figures, the MSN displays a population with a very small genotypic variance.

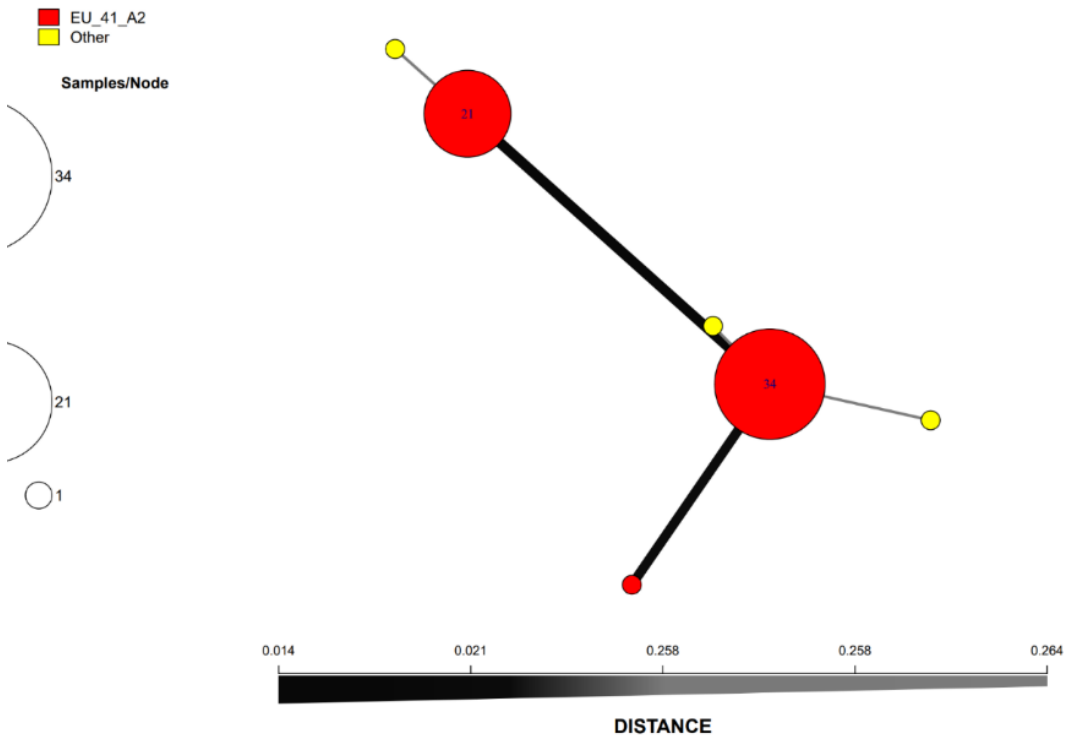

Figure 4. Minimum Spanning Network (MSN) of the collected samples based on Bruvo-distance. The colours of the circles represent if the isolate belongs to the clonal lineage EU_41_A2 or individual MLGs. Circle sizes are proportional to the number of samples where the largest circle includes 34 isolates, the medium one 21, while the small one contains one sample each. The MSN shows three different clades within the EU_41_A2 genotype.

3.2. Phenotypic variation: Fungicide sensitivity

3.2.1. Laboratory fungicide concentration

To examine if the chosen concentrations for the *in vitro* fungicide sensitivity tests gave the desired result the relative growth of all isolates on the three test concentrations were compared to the growth of the isolates on non-amended media (Fig. 5).

The result confirms that the chosen concentration of Infinito® amended on the plates in the lab test gave the expected result with almost no growth on the highest concentration and a growth reduction of approximately 40% in the medium concentration. However, the effect of the lowest concentration was too low; the treatment showed no reduced growth compared to control (Fig. 6).

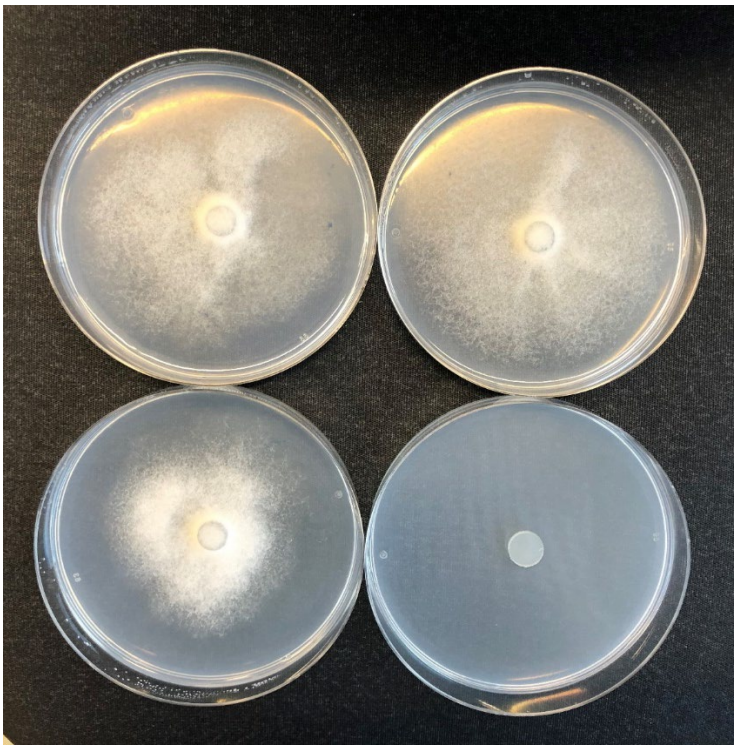

Figure 5. Result of the laboratory fungicide concentration. The agar plate to the right at the top is the control and the plate next to it is amended with the lowest concentration (0,1 ml Infinito). The plate to the bottom left is amended with the medium concentration (1 ml Infinito) and the last one with the highest concentration (10 ml Infinito). There is a clear reduction in the radial growth between the different concentrations.

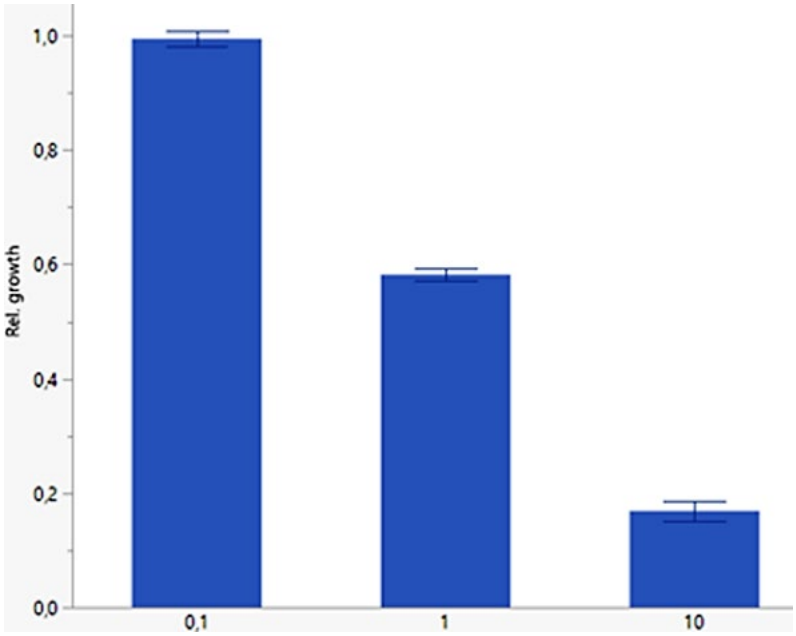

Figure 6. The mean growth of P. infestans for each of the three fungicide treatments compared to control: 0.1,1 and 10 mg/l agar. P <0,0001. Tukey-Kramer HSD: 0.1-A, 1-B, 10-C. Error bars represent standard deviation.

3.2.2. Fungicide tolerance testing

Sixty-one isolates were tested for fungicide tolerance since four of the samples were contaminated. Most of the isolates were from untreated plots, with a decreasing number of isolated with increased field concentrations (Table 2).

Table 2. Number of isolates from each field treatment used for testing of the phenotypic trait fungicide tolerance.

Field treatment	Number of isolates
Control	20
¼ dose	17
½ dose	13
Full dose	11
Total	61

The results from the *in vitro* fungicide tests showed no distinction between isolates from treated and untreated plots (Fig. 7). Nor did the result show any difference in the tolerance to fungicide when comparing the field treatments. There were no signs of selection towards more tolerant genotypes and no significant differences between individual isolates was observed.

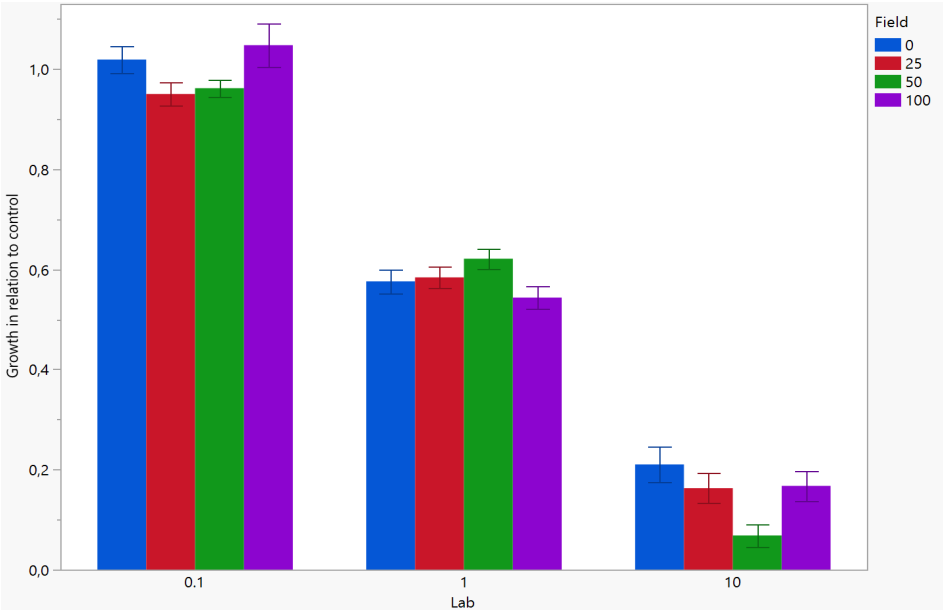

Figure 7. Growth related to untreated control for samples from the different field treatments (colours) on agar with different fungicide concentrations (grouping on the x-axis). P-values for the different lab treatments: 0.1 mg active substance/L agar: p=0,0613, 1.0 mg active substance/L agar: p=0,2031, 10 mg active substance/L agar: p=0,0710. Error bars represent standard error.

4. Discussion

4.1. Genotypic analysis

The result of the genotypic analysis in this study showed a surprisingly low genotypic diversity. The explanation for the low diversity was that most of the collected isolates belonged to the same clonal lineage, EU_41_A2, which was found in all field treatments. Within this clone, three clades could be distinguished based on genotyping with SSR markers, where two of these clades contained all but one of the isolates. The largest group contained 34 of the isolates while the second largest contained 21 of the isolates. This could mean that all the isolates in each of the EU_41_A2 clusters were re-sampling of isolates originating from two single infections and hence is the same "individual".

Since earlier studies made on Nordic populations has showed a great genetic diversity with a high number of unique SSR MLGs (Lehtinen et al. 2009; Brurberg et al. 2011; Sjöholm et al. 2013; Jonsson & Olsson 2015) the fact that a great majority of the collected isolates belonged to the same clone was very unexpected. Based on yearly sampling and genotyping, the Swedish sexual *P. infestans* population have been able to compete with the clones dominating in the rest of continental Europe and the UK. This can be attributed to a combination of several factors, where unfavourable climate for clonal overwintering of mycelia and oospores as an inoculum source are important.

4.2. Phenotypic variation: Fungicide sensitivity

Although the lowest lab concentration might have been too low, it is very unlikely that it had an effect of the result of the experiment. The dose-response curve showed a significant reduction in *in vitro* mycelial growth between all the tested fungicide concentrations.

The low genotypic variation indicates a low phenotypic variation in the studied field population, and this would limit the effect of selection. In a study similar to the one reported here, different fungicides were applied alternately at different dosages once a week (Jonsson & Olsson 2015), while in the current study the same fungicide was used all through the growing season. The use of a single fungicide should exert a very high selection pressure towards individuals expressing any level of reduced sensitivity to the specific fungicide. Despite of this, in the study by Jonsson & Olsson (2015) isolates collected from plots treated with high concentration of fungicides in field showed a reduced sensitivity to the fungicide *in vitro*, while in my study, no such correlations could be seen. The overall result of the study displays no signs of any reduced sensitivity to the fungicide used. No difference between the different field treatments regarding sensitivity to fungicides in laboratory could be seen. The relative growth of isolates from plots treated with full dose showed no significant difference from isolates collected from untreated plots.

A major difference between the two studies is the population structure. While the population in my study was dominated by a clone, and thus had a very low genotypic diversity, it was dominated by an oospore-derived population with a high genotypic diversity in Jonsson & Olsson (2015) study. For selection pressure to occur, a postulate is that the genetic trait is present in the population. In this population, with a majority belonging to the same clone, the results indicates that there is no resistance to "select towards". The diversity decreases further when considering the risk of many of the isolates belonging to the same individual.

Reports of failures to control late blight in potato is not uncommon and sometimes fungicide resistance is suspected as a cause of these observations. More likely is the lack of control the result of inadequate fungicide application, for example an inadequate spray coverage of the crop or an improper application timing. With *P. infestans* being a medium risk pathogen when it comes to the development of resistance to fungicides, this might be a likely explanation for the reduced efficacy in the studied field trial.

5. Conclusions

This study was done to examine whether there were any indications of selection from the use of different fungicide dosages in a field population of *P. infestans*. The hypotheses were based on that selection pressure exerted by fungicides leads to a lower sensitivity to fungicides in samples collected in treated plots compared to samples from untreated plots. Furthermore, we hypothesised that selection caused by fungicide use would cause a change in the genotypic population structure by higher rate of survival of less sensitive isolates compared to sensitive ones.

In this study, no difference was observed between isolates collected from treated plots and isolates collected in untreated plots. Furthermore, there were no indications that the dosage used in field had any effect on the tolerance to fungicides in vitro. Since most of the population belonged to the same clonal lineage, no change in the genotypic population structure could be observed.

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7. Populärvetenskaplig beskrivning

Potatis är, efter spannmål, den största grödan globalt. En kombination av relativt låg produktionskostnad, potentiellt höga skördar och ett högt näringsinnehåll leder till att den spelar en viktig roll som näringskälla i en stor del av världen.

Potatis kan drabbas av ett flertal sjukdomar där potatisbladmögel, orsakat av *Phytophthora infestans*, är en av de allvarligaste. Symptomen, som ofta börjar på bladen, karaktäriseras av mörka fläckar med död vävnad som snabbt sprider sig till hela plantan. Om vädret är gynnsamt för *P. infestans* kan hela fält utraderas inom en vecka. Sjukdomen kan även angripa knölarna som riskerar att drabbas av brunröta vilket kraftigt reducerar deras kvalitet.

Sjukdomen är svår att stoppa när den väl angripit grödan. För att skydda skörden används stora mängder växtskyddsmedel (fungicider) i förebyggande syfte. Användandet av fungicider är dessvärre förknippat med ett flertal nackdelar. En av dessa är risken att *P. infestans* utvecklar resistens mot preparaten som används vilket leder till att sjukdomen blir än mer svårbekämpad. Orsaken är att användningen selekterar fram de ”individer” i populationen som är minst känsliga mot fungiciden, medan resterande delen av populationen dör. De mer toleranta ”individerna” blir de som sprider sig, och bildar en mer tolerant population.

Syftet med detta examensarbete var att studera hur olika fungiciddoser påverkar populationsstrukturen i en fältpopulation av *P. infestans*. Detta var intressant att ta reda på då det under säsongen 2020 fanns indikationer på sviktande effekt av fungiciden Infinito® i ett fältförsök i södra Sverige. Blad infekterade med *P. infestans* från nämnda fält samlades in, och studerades i in vitro försök med olika fungiciddoser. Den genotypiska diversiteten bestämdes med hjälp av mikrosattelitmarkörer och populationsstrukturen och släktskap mellan de insamlade proverna analyserades

.

Resultatet visade inga tecken på reducerad känslighet mot fungiciden Infinito. Däremot visade resultatet en överraskande låg genotypisk diversitet där de flesta isolaten tillhörde samma klonala linje.

Den låga genotypiska diversiteten bland isolaten insamlade i försöket visade att vi fått in en konkurrenskraftig klon av *P. infestans* i Sverige vilket kan försvåra bekämpningen av sjukdomen framåt. Mer forskning på patogenens populationsstruktur är därför nödvändig.