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Evaluation of Detached Leaflet Assay as a High-Throughput Way to Select for Late Blight Resistant Potato

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Abstract

The potato (*Solanum tuberosum*) is the world's third most important food crop. It is grown all over the world. Late blight is an important disease in potato, caused by the pathogen *Phytophthora infestans*. The disease causes large crop losses and infects both the foliage and tubers, if not controlled by fungicide application. Developing new potato cultivars with improved resistance against late blight, is important in breeding programs, as these would allow less frequent spraying and a more sustainable potato production. Resistance against foliage late blight is usually evaluated in large scale field trials. Such trials are often expensive, difficult to manage and control. Alternative ways of assessing resistance that is cost-effective, high-throughput and precise are therefore of great interest.

The purpose of this thesis is to investigate whether a detached leaflet assay (DLA) can be used to detect resistance against foliage late blight in different potato genotypes. This was investigated through assessment of 255 cultivars and breeding material (genotypes) representing the Norwegian germplasm. One plant per genotype was grown in pots in a greenhouse. After 8 weeks, leaves were detached and 9 leaflets per genotype were placed in Petri dishes and inoculated with *P. infestans*. The infection was assessed visually and by image analysis 6 and 8 days after inoculation. The results were compared with historical field data, and a genome-wide association study was performed.

The results showed that the leaf test was able to distinguish potato genotypes based on their susceptibility to late blight. There was a low frequency of infection on the leaflets. A reason for this may be that the inoculum had reduced infectivity due to age. The correlation between the DLA and the historical field data was limited, but significant. The leaf test was able to identify the potato genotypes most susceptible to late blight. The association study found several significant markers. A marker found on chromosome 3, was located near a known QTL for late blight resistance.

It can be concluded that the DLA can be used to identify the genotypes that are most susceptible to foliage late blight before an eventual field trial.

Sammendrag

Potet (*Solanum tuberosum*) er verdens tredje viktigste matplante. Den dyrkes over hele verden. Tørråte er en viktig potetsykdom, og forårsakes av skadegjøreren *Phytophthora infestans*. Tørråte kan føre til store avlingstap og kan angripe både riset og knollene om det ikke sprøytes jevnlig. Utvikling av nye potetsorter med forbedret motstandsdyktighet mot tørråte er viktig i potetforedling, ettersom slike potetsorter kan føre til sjeldnere sprøytninger og en mer bærekraftig produksjon. Resistens mot tørråte på ris vurderes vanligvis i feltforsøk. Feltforsøkene er ofte kostbare, vanskelige å administrere og kontrollere. Alternative måter for å teste tørråteresistens som både er presise, tidseffektive og kostnadseffektive er derfor av stor interesse.

Hensikten med denne oppgaven er å undersøke om en bladtest kan benyttes til å avdekke resistens mot tørråte på ris blant potetgenotyper. Dette ble undersøkt ved hjelp av 255 ulike potetsorter og foredlingskloner (genotyper) som representerer arvemassen til norsk potet. En plante per genotype ble dyrket i potter i et drivhus. Etter 8 uker ble blader høstet inn og 9 småblad per genotype ble plassert i Petriskåler og inokulert med *P. infestans*. Infeksjonen ble vurdert visuelt og ved bildeanalyse 6 og 8 dager etter inokulasjon. Resultatene ble sammenlignet med historiske feltdata, og det ble utført en genomvid assosiasjonsstudie (GWAS).

Resultatene viste at bladtesten klarte å skille mellom potetgenotypene basert på deres mottakelighet for tørråte. Det var i utgangspunktet få blad som ble infisert. En årsak til dette kan ha vært at smitteløsningen var lite smittsom på grunn av alder. Korrelasjonen mellom bladtesten og de historiske feltdataene var begrenset, men signifikant. Bladtesten klarte å identifisere potetgenotypene som var mest mottakelige for tørråte. Assosiasjonsstudien fant flere signifikante markører. En markør funnet på kromosom 3, var lokalisert nær en tidligere omtalt kvantitative arveegenskap (QTL) for tørråteresistens.

Det kan konkluderes med at bladtester kan benyttes for å identifisere genotypene som er aller mest mottakelige for tørråte på ris før et eventuelt feltforsøk.

Abbreviations

AUDPC	Area under the disease progress curve
AULEC	Area under the lesion expansion curve
cm	Centimetre
d	Diameter
DLA	Detached leaflet assay
ETI	Effector triggered immunity
g	Gram
GWAS	Genome-wide association study
ha	hectare
K	Kelvin
L	Litre
Mb	Million base-pair
ml	millilitre
NIBIO	Norwegian Institute for Bioeconomy Research
PAMP	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
QTL	Quantitative trait locus
R	Resistance
SNP	Single nucleotide polymorphism
W	Watt

Contents

1. Introduction

1.1. Potato

1.1.1. Origin and history

Potato (*Solanum tuberosum*) is a versatile crop, and one of the world's most widely grown crops. It belongs to the *Solanaceae* family together with tomato (*Lycopersicon lycopersicum*), bell pepper (*Caspicum annuum*), cultivated tobacco (*Nicotiana tabacum*), aubergine (*S. melongena*) and 3000-4000 other species. The nearest relatives are other tuber-bearing *Solanum* species, classified as *Solanum* section *Petota*, with 107 different species *(Spooner et al., 2014)*. The cultivated potato has a monophyletic origin that can be traced back to a single domestication event of the species *S. candolleanum* or one its descendants (Spooner et al., 2005). The many and diverse landraces that are found in Andean and Chilean region are thought to be the result of subsequent crosses and not independent origins (Jansky & Spooner, 2018). Dating the domestication of potato is difficult, but Bradshaw (2021) cited archaeological evidence that dated to 7000 and 5420-3620 years before present time. The crop became an important part of the diet for people in Andean societies around 2000 years ago (De Jong, 2016).

Colonization of South America by Europeans resulted in potato tubers being transported across the Pacific Ocean to Europe. The first written record of potato in Europe is from Spain in 1573 (Hawkes & Francisco-Ortega, 1993). Cultivation was limited the first hundred years as the crop was unknown and the environmental differences between South America and Europe affected tuberization (Gutaker et al., 2019). Later, potato gained importance as a food crop as an alternative to cereals against famines and allowed a large increase in the European population (Ames & Spooner, 2008). In comparison to cereal, it could provide once or twice more calories per hectare. It was a sturdy crop providing consistently good yields (Zadoks, 2008). As an established food crop in Europe it was exported to the rest of the world by the European colonists, and is today an important crop in all continents (De Jong, 2016).

1.1.2. Production and uses

Potato is cultivated on a total area of 16.5 million hectares with an annual production of 359 million tonnes as in 2020, making it the third most important food crop after wheat and rice (FAOSTAT, 2022). Historically potato production has been bigger in the developed countries in Europe and North America, than in developing countries. However global production has shifted over to developing countries in Asia and Africa, where the largest increase in production has been seen the last 20 years (Devaux et al., 2020).

Potato is an important crop in Norway with an average annual production of 337 000 tonnes in the last 5 years (Statistisk sentralbyrå, 2022). In comparison, the neighbouring countries Finland, Sweden and Denmark produced 624 000 tonnes, 879 000 tonnes and 2 763 000 tonnes, respectively, in 2020 (Eurostat, 2021). There has been a steady decline in area used for potato production in Norway, from 15 239 ha in 2001 to 12 896 ha in 2011 and, currently, 11 433 ha in 2021 (Statistisk sentralbyrå, 2022). The decline could be a result of reduced demand for potato from consumers (Helsedirektoratet, 2022). Consumption per capita is now 52 kg, comparing to 100 kg in the 1950s. The consumption of table potatoes has decreased while the use of processed products, such as French fries, chips and mashed potatoes, have increased.

1.1.3. Cultivation in Norway

Close to 77 % of the potato area in Norway is situated in Eastern Norway, but there are production throughout the whole country (Møllerhagen, 2022). Due to the short growing season, some farmers pre-sprout seed potatoes before planting. This advances the emergence and shorten the growth cycle, allowing farmers to sell at a premium early in the season or ensure that the potatoes mature before the autumn frost (Mølmann & Johansen, 2020). Row covers are also used by some farmers to protect against night-time frost early in the season. As potato is clonally propagated it can accumulate many diseases when grown over multiple years, this is especially true for viruses. Norway has a highly functional seed potato production system that provides certified disease-free material for the farmers (Aspeslåen et al., 2016). In 2021, 39 % of the seed tubers used by farmers were certified (Møllerhagen, 2022). The use of healthy seed tuber is important in the management of many diseases.

The cold and humid climate in Norway is favourable for the development of many potato diseases. Both the foliage, stems and the tubers of the potato crop can be targets for disease. In

the growing season the foliage is susceptible to pests and pathogens. Soil-borne diseases can affect the roots of the plant and tubers. During harvest the infected foliage can come in contact with the tubers and transfer disease. In storage, postharvest diseases can infect and rot away the tubers. The main potato disease in Norway is potato late blight (Hermansen et al., 2012). This disease can spread quickly over a field if left unmanaged. Infections in foliage will result in a yield decrease as leaves die, and all aboveground parts of the plant eventually rot away. Infected tubers show brownish blotches that extends into the flesh of the tuber. The rot can develop during storage and allows secondary pathogens to infect the tuber (Agrios, 2005). Tuber infection are of great concern, as some potato-processing industries and most potato-packing companies have a 'zero tolerance' to tuber blight, leaving the farmer with no income from his potatoes (Hermansen et al., 2012).

1.2. *Phytophthora infestans*

The oomycete *Phytophthora infestans* is the pathogen responsible for late blight disease in potato. The pathogen has the ability to reproduce efficiently and can ravage a potato field in days. It is of historic importance as its epidemics in Europe in the 1840s resulted in the Great Irish famine with ~600 000 excess deaths and emigration of ~1 300 000 people from Ireland in a five-year period (Zadoks, 2008). The Irish people were heavily dependent on potato as a chief staple, so when the harvest failed the consequences were catastrophic. The late blight epidemic also affected continental Europe, and at the same time yields of other staple crops such as rye were reduced due to rust, frost and drought, resulting in an estimated 700 000 hunger-related deaths (Zadoks, 2008). Since then, *P. infestans* has been a constant threat in countries with potato production, and it is estimated that it is responsible for an annual loss of €5.2 billion due to crop losses and cost of control measures (Haverkort et al., 2009).

P. infestans belongs to the class of oomycetes. Oomycetes are eukaryotic organisms phylogenetically related to brown algae and diatoms in kingdom Chromista (Cavalier-Smith, 2018). Due to their ability to produce spores and filamentous growth in their vegetative stage they were earlier wrongly classified in the kingdom of Fungi. Oomycetes are different from fungi in several important traits: their cell-walls primarily consist of glucan and not chitin as in fungi; the lysine pathways are different; they produce zoospores with two flagella while the fungi that produce zoospores have one; they are typically diploid in the vegetative stages of their life cycle (Fry & Grundwald, 2010; Thines, 2014). Potato and tomato are the two most economically important hosts of *P. infestans*, but wild species of solanaceous plants can also be infected (Lindqvist-Kreuze et al., 2020).

The centre of origin of *P. infestans* is heavily debated and not certain. The two main hypotheses are that it either originates from central Mexico or South American Andes, with new arguments constantly being published supporting both sides (Goss et al., 2014; Martin et al., 2016). In the discussion about *P. infestans*, strains and lineages are often used to describe specific genotypes of the pathogen as it normally propagates clonally. For example it has been shown that the lineage HERB-1 caused the epidemics in Europe in the 1840s after being spread from North America, but that this lineage later were outcompeted and replaced by a new lineage, US-1, that were the dominant lineage in the world until the end of the 1970s (Yoshida et al., 2013). The introduction of a new lineage into an area can result in difficulties as the new lineages have different characteristics. New lineages can be more aggressive and be pathogenic to previously resistant cultivars.

P. infestans is heterothallic. Historically, sexual reproduction of the pathogen only occurred in Mexico, the only place where both mating types were present. Outside Mexico the populations only consisted of the A1 mating type and could only reproduce asexually. However this changed in the 1970s when potatoes from Mexico likely containing strains of the A2 mating type were imported to Europe (Fry, 2020). Sexual reproduction is likely beneficial for the pathogen and make late blight management more difficult. In the Nordic countries and Northwest Russia, the majority of *P. infestans* populations are now sexually recombining resulting in genetically diverse population (Brurberg et al., 2011; Puidet et al., 2022; Runno-Paurson et al., 2022). In contrast, clonal lineages are still dominating in western Europe and USA despite both mating types being present (Fry, 2020; Mariette et al., 2016).

1.2.1. Life cycle

P. infestans can reproduce both asexually and sexually, both cycles are depicted in Figure 1. As mentioned earlier, the presence of both mating types is required for the sexual reproduction since the organism is heterothallic. When mycelia of the two mating types meet in the host tissue, they can form gametangia, and the antheridium of one mating type fertilizes the oogonium of the other. This results in the formation of a thick-walled oospore. Oospores are dormant and can survive for a long time before they eventually germinate and form a sporangium. Sporangia are also formed asexually on mycelium developing into sporangiophores. The sporangia can be carried with the wind or water to new parts of the plant or other host plants. At temperatures below 14°C, sporangia release 3-8 zoospores that can swim short distances to the host, while at higher temperatures sporangia germinate directly and form a germ tube that can colonize the host through wounds or natural openings (Judelson & Blanco, 2005). When zoospores have located the host, they will encyst and form an appressorium that can penetrate the plant epidermis. Most infections occur through zoospores (Kessel et al., 2009). After initial infection, hyphae will spread throughout the plant and form feeding structures, haustoria. Infection starts with a biotrophic phase, but later the pathogen switches to necrotrophic growth and necrotic lesions will become visible. Under the right conditions, in cool and wet weather, it can take only 4 days before new sporangia are formed on sporangiophores in a susceptible host and the cycle is repeated (Kamoun et al., 2015).

Tubers, foliage, and stems of the potato plant can all be infected by *P. infestans*. With the short generation time the disease can quickly spread in a field under the right conditions, which is

high humidity and temperatures between 15-25°C (Agrios, 2005). The pathogen can enter a field through infected tubers or through the air as sporangia. On cloudy days sporangia can survive for hours in the atmosphere and travel long distances in the air (Mizubuti et al., 2000). Oospores are also a possible source of primary inoculum. The sexual and highly diverse *P. infestans* population in the Nordic countries produce oospores that can survive the winter (Cooke et al., 2011; Puidet et al., 2022). On foliage typical symptoms are brown necrotic lesions surrounded by white mycelium with sporangiophores. Sporangia from leaves can then be washed down and zoospores can infect the tubers, causing brown sunken areas (Agrios, 2005).

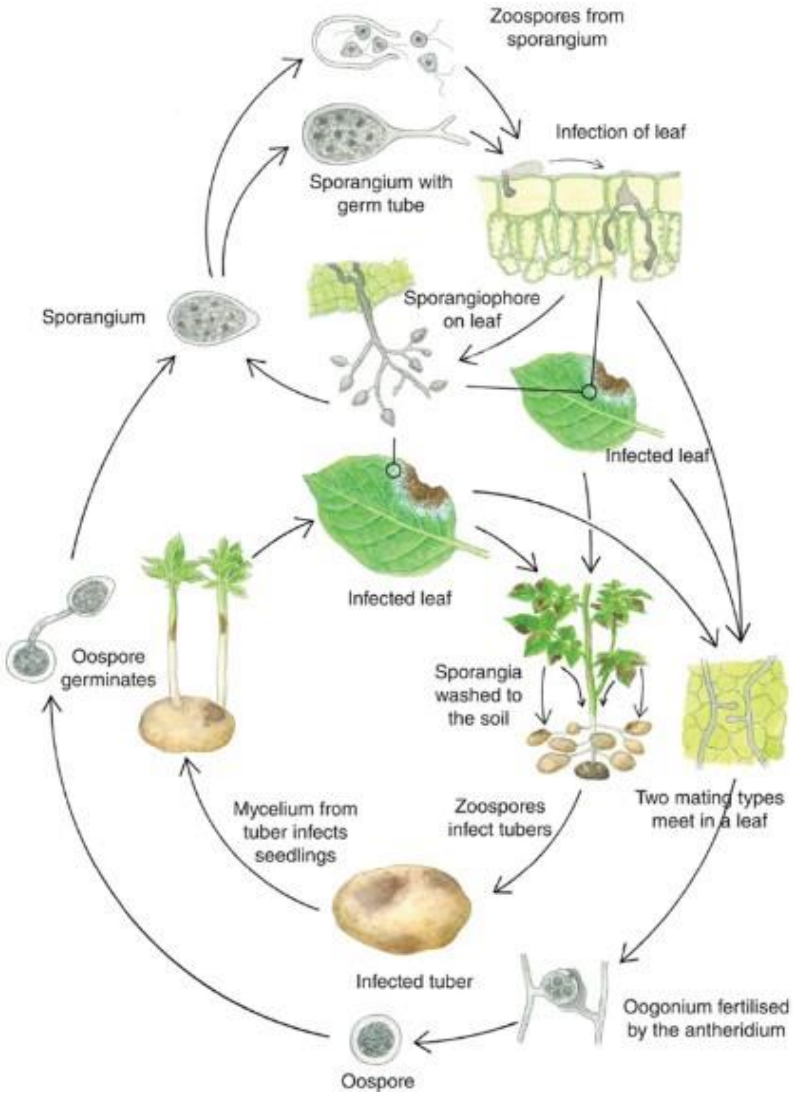

Figure 1. Disease cycle of *Phytophthora infestans* on potato. The inner circle depicts the asexual stage, while the outer circle shows the sexual stage. Further details are given in the text. Adapted from Tronsmo et al. (2020), drawing by H. Karlsen.

1.2.2. Strategies for management of late blight

Successful control of late blight can be achieved through a combination of agricultural practices. It is important to reduce all possible sources of primary inoculum. Infections early in the season are especially problematic as they require repeated fungicide treatment throughout the season. Only disease-free tubers should be used as seed, and cull piles should be buried or covered with enough soil. Volunteer plants outside the field that will not receive fungicide applications should also be removed. Crop rotation is helpful against oospores, although it uncertain how long they can survive in the soil and pose a risk as primary inoculum (Puidet et al., 2022). Hilling of potatoes before canopy closing will reduce the chance of spores from the foliage from reaching tubers as the water is more likely to run off between the furrows rather than down to the tubers. Haulm toppers and chemical desiccants are used to kill of foliage and stems before harvest, so that infected tissue does not come in contact with tubers and transfer disease during harvest. Chemical pesticides against late blight are sprayed repeatedly during the season in Norway and in some regions more than 8 times per season (Hjelkrem et al., 2021). The majority of fungicides against late blight are protectant and should be applied to the crop shortly before infection would occur. Farmers can use forecast models as a tool to decide when to spray (Hjelkrem et al., 2021). In Norway there is a freely available decision support system available for farmers. The model combines information about late blight observations, weather data and weather forecast data to predict the risk of late blight infections (Brodal et al., 2016). Using potato cultivars with resistance against late blight is another important strategy. The resistance of a cultivar will depend on the pathogen population, but some possesses partial resistance effective against all pathogen populations, often called field resistance. The spraying interval can be extended if the cultivar has high level of resistance, which would be in accordance with the political push to reduce pesticide use in agriculture (European Commission, 2020; Nærstad et al., 2007). Breeding new cultivars with improved resistance is therefore crucial.

1.3. Plant defence and resistance

The plant itself is not defenceless against pathogens. Niks et al. (2019) assigned plants three defence strategies: avoidance, tolerance, and resistance. In avoidance the contact between the plant and the pathogen is reduced. Plants can make use of camouflage or chemical repellents against herbivores, but since pathogens reach plants passively these kinds of avoidances do not help. On the other hand, crop architecture can have a role. Fewer spores are able to land on erect leaves compared to horizontal leaves, and a more open canopy will give a different micro-climate than a closed one. In potato, the infection of tubers in the field can depend on how the tubers are distributed in the ridge and how the canopy either transport the water along the stem allowing transport of spores to tubers or shed the rainfall between ridges (Nærstad et al., 2007). Tolerance can be defined as the capacity of the plant to restrict the amount of damage or symptoms per unit of quantity of pathogen present (Niks et al., 2019). A tolerant plant will be less damaged by a pathogen than a sensitive plant. Resistance is the ability of a plant to reduce the development and/or growth of the pathogen after contact has been established (Niks et al., 2019). A plant can be resistant due to a general defence of physical barriers or preformed inhibitors and/or it can induce defence responses that activate when the pathogen is recognized.

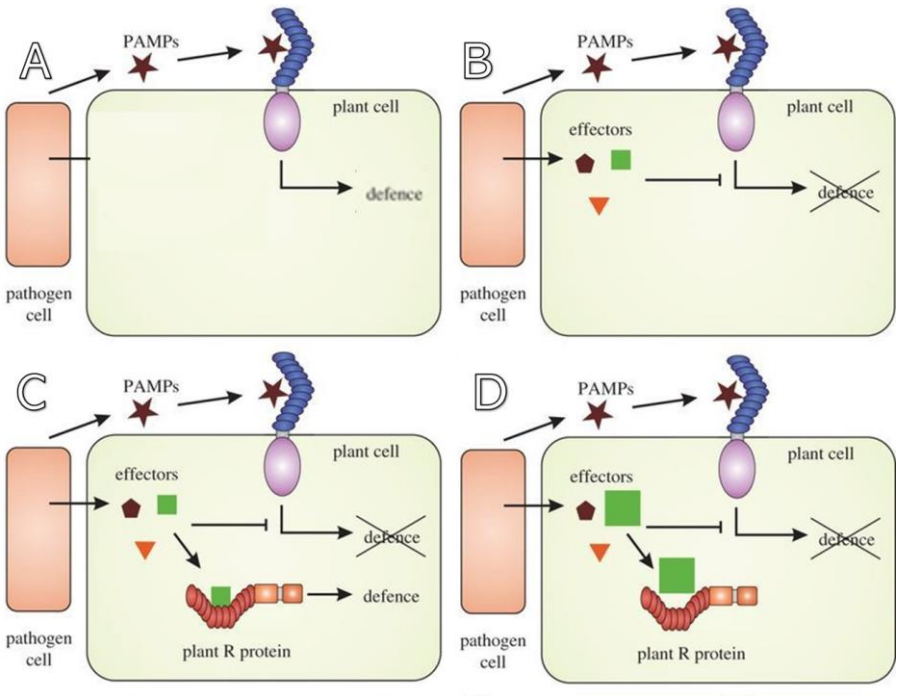

Figure 2. Model of plant-microbe interactions. **A.** Recognition of a pathogen-associated-molecular-pattern (PAMP) triggers immune responses in the plant cell. **B.** The microbe secretes effectors that inhibit the defence signalling. **C**. Resistance (R) protein recognizes an effector from the pathogen resulting in an effector-triggered immune response. **D.** The pathogen can evolve and gain effectors that are not recognized by the R protein, resulting in infection. After Jones et al. (2014).

The inducible defence that plants possess can be explained through models described in Figure 2. Biochemical compounds, often called pathogen-associated molecular patterns (PAMP), can be perceived by plant receptors that will initiate a signalling sequence resulting in immune responses, often named PAMP-triggered immunity (PTI). The immune responses can activate a defence system. In order to overcome this defence system, pathogens can secrete effector molecules into the plant cells. The effectors can interfere with the signalling sequence involved in the PTI, leaving the plant cell unable respond to the infection. However, some plants have evolved the capacity to recognize effectors from pathogens. Recognition is often mediated by intracellular receptors, resistance (R) proteins (Jones et al., 2014). After recognition of an effector, the R protein will often initiate an immune response and confer resistance. This is often called effector triggered immunity (ETI). Through mutation or loss of the recognized effector, the pathogen can evade recognition, not triggering ETI, and once again successfully infect the plant cell (Jones et al., 2014). This is what happens when resistance "break down".

The effectors that pathogens secrete are specific and affect a particular target in the plant cell. This specialization is why pathogens often have a limited host range (Niks et al., 2019). The PTI responses constitute a defence against non-specialized pathogens without any effectors. With the specificity of effectors and ETI, not all genotypes of the pathogen or the host in a population have the same ability to cause disease or initiate defence responses. The plant resistance protein in Figure 2 is race-specific as it only recognizes the pathogen in panel C and not in panel D. The pathogen in panel C has an avirulence (Avr) effector that is recognized by the R protein resulting in a defence response, while the pathogen in panel D has a virulence effector (avr) not recognized by the R protein resulting in a lack of defence response (Flor, 1971). Several R proteins and corresponding avirulence effectors have been described in potato and *P. infestans* (Vleeshouwers et al., 2011).

1.4. Breeding of potatoes

1.4.1. Genetics of potato

The cultivated potato is autotetraploid with a basic chromosome number of 12 (2n = 4x = 48). Its wild relatives and cultivated landraces have ploidy levels ranging from diploidy (2x) to hexaploidy (6x) (Bradshaw, 2021). The cultivated potato is highly heterozygous, outcrossing and with tetrasomic inheritance which complicates genetic analysis. While selfing of a heterozygous diploid (Aa) would result in three genotypes (AA, Aa, aa), a similar selfing of a tetraploid (AAaa) would result in five genotypes (AAAa, AAAa, AAaa, Aaaa, aaaa). Double reduction is a phenomenon that occur during meiosis due to tetrasomic inheritance and will affect the mendelian ratio of a cross. The result is two sister chromatids ending up in the same gamete (Ortiz & Mihovilovich, 2020). Epistasis, the phenomenon where the expression of one gene is modified by the expression of other genes, is common.

The genetic base of potato in Europe was previously quite narrow due to few introductions of new genes from wild species and landraces. However, there has been a great effort in introducing new germplasm, particularly with resistance against diseases and pests (Hawkes, 1978; Srivastava et al., 2016). The interesting genes and traits of wild species can be closely linked to undesirable genes. This is called linkage drag. Multiple backcrosses are needed to break this linkage, so that the final contribution of the wild species' genome is minimal. There are reproductive barriers between wild species and cultivated potato. This is due to differences in ploidy and endosperm balance number. Breeders are able to overcome these barriers using ploidy manipulations and bridge crosses (Jansky, 2006).

1.4.2. Breeder's equation

The aim of breeding is improving traits through genetics. A popular equation to describe how different parameters affect the speed of this improvement is the "breeder's equation", where the Genetic gain over time (R_t) is a product of selection intensity (i), selection accuracy (r) and additive genetic variation within the population (σ_A), divided by the numbers of year per cycle (t) (Cobb et al., 2019).

$$R_t = \frac{i * r * \sigma_A}{t}$$

In potato breeding, a breeding cycle starts with a cross and the progenies (true potato seeds) are sown out. Later the genotypes are clonally propagated through tubers and evaluated for traits over multiple years. Selection intensity (i) is how much the mean of the selected genotypes deviate from the population mean. This parameter is affected by the population size, amount of discarding and selection speed. A larger population will increase the costs of the breeding program, since more material must be evaluated and handled. On the other hand, discarding more material and faster selection will decrease costs. The selection accuracy (r) depends on how many replicates are tested and the precision of the testing method. Multiple replicates and more precise evaluation will increase the accuracy and costs. Balancing cost and results are important in a successful program.

1.4.3. Potato breeding in Norway

Potato breeding in Norway is conducted by Graminor AS. Potato breeding in Norway can be traced back to 1920 and Norges Landbrukshøgskole (NLH), today Norwegian University of Life Sciences (NMBU), in Ås. The history of the potato breeding program until 2008 is covered in Bjor and Bundgaard (2008). Briefly, the program was from 1920 until 1998 a part of the research and development activity at NLH. In 1998, it was transferred to Planteforsk, today a part of Norwegian Institute of Bioeconomy Research (NIBIO) and ran in close collaboration with NLH. In 2003, the program was transferred to Graminor, a breeding company established in 2002 to unite Norwegian plant breeding in a single company. As a result of this, the potato breeding program relocated to Bjørke, close to Hamar, in 2006. Graminor (www.graminor.no) is owned by the Norwegian state; the agricultural cooperatives Felleskjøpet Agri SA, Gartnerhallen SA and Strand Unikorn AS; and Graminor AS itself. The program has been led by Dr. Aksel P. Lunden (1920-1964), Dr. Lars Roer (1964-1990), Dr. Tore Bjor (1990-2006), Ms Kirsten Bundgaard (2004-2013), and Dr. Muath Alsheikh (2013-present).

The potato breeding program is rather small scale. The aim of the program is to develop new potato cultivars suited for the Norwegian environment. The Fennoscandian region, including Norway, has short and intense seasons with long day length, specific consumer preferences, and specific pathogen pressure (Eriksson et al., 2016). The program breeds cultivars both for fresh consumption (table potatoes, salad, and baking) and processing (French fries and chips). Both types of cultivars are weighted equally in the program.

The breeding starts by selecting suitable parents for crossing. Crosses are done in inside a tent in the greenhouse to prevent insects from pollinating. The resulting true potato seeds are sown out in seed trays, and the ones that germinate are transplanted into single pots after two weeks. Typical numbers of genotypes per cycle for the first years after a cross are: 100 crosses result in 25 000 true potato seeds in year 0; 20 000 seedlings in greenhouse producing mini-tubers in year 1; 17 000 genotypes in year 2; 1500 genotypes in year 3; 200 genotypes in year 4. To evaluate maturity in higher latitudes, tubers of genotypes that are considered potential table potatoes are evaluated in plots in Northern Norway in year 2.

In the first years, the genotypes are mainly selected during harvest based on the visual appearance of tubers. It is important that the tuber shape is agreement with the intended segment of the genotype, e.g., small, round for salad, or long-oval for French fries. Tuber size

distribution and equalness are also considered. In years with weather conditions conducive to common scab development, it also possible to select for resistance to *Strepomyces* spp. Genotypes are tested more thoroughly in the subsequent years.

In year 5 to 9 the genotypes are tested for yield, disease resistance and quality traits. The genotypes are continuously evaluated and abandoned if they perform poorly. In yield trials the genotypes are compared to standard cultivars. External yield trials are conducted in six different locations in potato producing regions in Norway. The genotypes are tested for resistance against late blight on foliage and tubers (*P. infestans*), common scab (*Streptomyces* spp.), gangrene (*Phoma foveate*), Fusarium dry rot (*Fusarium* spp.), golden potato cyst nematode (*Globodera rostochiensis*) and wart (*Synchytrium endobioticum*). Tested potato quality traits depend on the intended segment of the genotype, but dry matter content, dormancy period and quality after washing are tested for all genotypes. Genotypes go through these tests at least three years in order to provide data for selection. In the end, tubers of the most promising genotypes, usually 3–5, are sent to the NIBIO Plant Clinic for in vitro culturing and later to Overhalla Klonavlsenter where they propagate clean tubers for official testing.

Before a new genotype can become a cultivar it has to go through a VCU-test (value for cultivation and use) for three years (Plantesortforskriften, 1999). Genotypes that are kept after the first year of VCU-testing will also go into a DUS-test (distinctness, uniformity, and stability). The Norwegian Food Safety Authority has assigned NIBIO Apelsvoll the responsibility of these tests. The tests and their results are described in detail in Møllerhagen et al. (2022) and previous publications of the Jord- og Plantekultur series. In addition to previous resistance tests, resistance against rust from Potato mop-top virus and Tobacco rattle virus, and against white potato cyst nematode (*G. pallida*) and more pathotypes of golden potato cyst nematode, are evaluated. The genotypes are currently not tested for resistance against Potato virus Y, blackleg/ soft rot (*Pectobacterium* spp.), silver scurf (*Helminthosporium solani*) and black scurf (*Rhizoctonia solani*). Genotypes can be retracted from the testing by Graminor any year. Genotypes that achieved good results can be applied for release as a new cultivar. Some recently released cultivars are ‘Hassel’ (2018), ‘Nansen’ (2018), ‘Birkeland’ (2021), ‘Gullflaks’ (2021) and ‘Knallfiffi’ (2021).

1.4.4. Breeding for disease resistance

Breeding of new cultivars with improved resistance to diseases and pests is an important objective. The relationship between a pathogen and the defence mechanisms of a plant is described in an earlier section. It is possible to find genes involved in resistance against the most important pathogens in released cultivars, breeding material, and wild populations of potato or one of its relatives. However, crossing in resistance (R) genes is not necessarily a durable solution. In similar fashion to how breeders can evolve the potato, the pathogen can evolve and overcome the new genetic resistance. The evolutionary potential of pathogen populations can be predicted and this information can be used to breed for durable resistance (McDonald & Linde, 2002). The five evolutionary forces, mutation, genetic drift, gene flow, reproduction, and selection, and their interactions are important.

P. infestans produces asexual spores that can spread over distance in the air, its effector genes are in dynamic and expanded genomic regions with high possibility of mutations, has two mating types allowing for sexual reproduction and oospore overwintering, and a large pathogen population as potato and other hosts are major crops, resulting in a high evolutionary risk (Haas et al., 2009; McDonald & Linde, 2002). The high evolutionary risk of the pathogen makes durable race-specific resistance an unlikely achievement, therefore non race-specific resistance, also called field resistance, should be prioritized (McDonald & Linde, 2002). However, in distant regions with only few clonal lineages of *P. infestans* and no sexual reproduction, such as sub-Saharan Africa (Pule et al., 2013), it could be feasible (Ghislain et al., 2019). To evade deploying new R genes that quickly will be defeated by the *P. infestans*, Vleeshouwers et al. (2011) advocate for assessing the role of the avirulence effector in *P. infestans* that are being targeted by the R gene product. Spending effort breeding new cultivars with R genes that the pathogen easily can overcome by getting rid of a dispensable effector is ineffective. On the other hand, there are notions that R proteins targeting indispensable effectors could be durable (Jones et al., 2014).

An effective and reliable way of testing new genotypes for resistance is important. A breeding cross will result in progenies with different degrees of resistance. Plants can be tested in the green house, in the laboratory or in the field (Niks et al., 2019). Field tests have growing conditions that resemble the commercial cultivation but cannot be controlled the same way as the other two methods. Field tests depend on conditions in the growing season such as temperature, irradiance, and drought, and if something goes wrong you will have to wait until

the next year. The pathogen must be evenly distributed in field so that the disease pressure is similar for all genotypes. Irrigation and susceptible spreader plants are used to ensure this. The fields can be sprayed with herbicides, pesticides and fungicides to ensure that the conditions are equal and that no other factor than *P. infestans* affects the result. Replications will increase the certainty of the result. An important strength of fields tests is that *P. infestans* will be polycyclic and go through multiple cycles of spread and infection. In comparison, greenhouse and laboratory tests, usually, are only designed as monocyclic tests. The pitfall of greenhouse and laboratory tests are that their results are not always representative for what happens in the field. Plants may be subject to more optimal growing conditions in greenhouses, but they can be more susceptible. Greenhouse and laboratory tests give possibilities to target the growth stage of the plant, inoculum dose, incubation environment and assessment method. In laboratory tests with detached leaves or leaflets it is important to make sure that the detached part is representative for the reaction of the whole plant (Niks et al., 2019).

Field tests with *P. infestans* is often evaluated by assessing the infection in field multiple times and then using these repeated observations to calculate the area under the disease progress curve (AUDPC). If needed, AUDPC value can be converted to a 1-9 resistance score through a quadratic regression model with the help of standard cultivars (Hansen et al., 2007). However, it is bold to summarise the resistance into just a single score and it is important to remember all the factors involved. Genotypes will affect these factors differently and it is the sum that ultimately amount to the resistance seen. Umærus (1963) listed the following factors that affect field resistance: entrance of the parasite, rate of growth of mycelium, rate of necrosis formation, generation time, sporulation capacity, nature of leaf structure, and growth habit of the plant. Some attributes contributing to variation in late blight reaction between genotypes in the field; e.g. growth habit, canopy density and structure, and nature of leaf surface; will not impact the results in laboratory tests, possibly leading to variation between laboratory and field assessments (Singh & Birhman, 1994).

1.5. SNP genotyping and GWAS

A genetic marker is a nucleotide sequence where a polymorphism can be detected between different individuals (Selga, 2021). In our experiment we use single nucleotide polymorphisms (SNPs) markers. Mutations have led to polymorphism in single base-pairs, and these can be found throughout the potato genome. A study found a variant density of 1 SNP/ 24 base-pair in exons and 1 SNP /15 base-pair in introns (Uitdewilligen et al., 2013). Next-Generation Sequencing of DNA has led to the discovery of many SNPs in potato. SNP arrays for quick and cost-effective genotyping has been developed from the discovered SNPs (Bradshaw, 2016). Examples of these arrays are the 20K SolSTW (Uitdewilligen et al., 2013; Vos et al., 2015) and 8K SolCAP arrays (Felcher et al., 2012; Hamilton et al., 2011). The first were developed using European potato germplasm, while the other used North American potato germplasm. SNP arrays are limited by the heterozygosity of the panel of genotypes used in the development of the array (Geibel et al., 2021; Selga et al., 2022). As an example, only 50% of the SNPs of the 8K SolCAP array were informative in the analysis of International Potato Center (CIP) germplasm (Lindqvist-Kreuze et al., 2021). Therefore, a new array, adapted to the higher introgression from the South American gene pool, were constructed (Lindqvist-Kreuze et al., 2021).

The genotype information can be used in analyses to identify quantitative trait loci, QTLs. The contribution of a single QTL to a trait will vary. Some contributions are so small that they are difficult to detect, while others contribute significantly and are easier to detect (Bradshaw, 2021). Genome-wide association study (GWAS), or association mapping, can be used to find SNP markers positioned closely to QTLs. All genotypes in the analysis are genotyped and assessed for the traits in interest. A benefit of GWAS is that the studied genotypes do not have to be related, this is contrast to biparental QTL mapping where all genotypes are from the same cross (Hackett et al., 2014). As a result of this, discovered marker-trait associations in a GWAS are more likely to be robust across a wider germplasm pool (Sharma et al., 2018). A sufficient marker density is necessary to discover QTLs through GWAS. The highest estimate is 3 million SNPs (Stich et al., 2013), but a later study found 40 000 SNPs as an upper bound and that 15 000 SNPs can be sufficient (Vos et al., 2017). Selga et al. (2021) propose a pipeline to reduce the number of SNPs and thereby genotyping cost, while retaining enough genetic variation for analysis. Correcting for population structure among the genotypes in a GWAS is important in order to avoid false positives (Sharma et al., 2018).

There are multiple software available that can be used to run a GWAS. For potato, the R package GWASpoly (Rosyara et al., 2016) is currently the most popular and used by many authors (Aliche et al., 2019; Kaiser et al., 2020; Koizumi et al., 2021; Lindqvist-Kreuze et al., 2021; Selga et al., 2021; Sharma et al., 2018; Yousaf et al., 2021; Yuan et al., 2020; Zhang et al., 2022). GWASpoly uses biallelic SNPs, so in an autotetraploid such as potato there are five possible genotypes classes for a SNP. The classes can be parameterized by the dosage of the minor allele: 0, 1, 2, 3, 4. The software supports multiple gene action models. In the general model the fixed effect for each genotype class is arbitrary. The other models are single-parameter genetic models: additive, simplex dominant, duplex dominant, and diploidized additive, all depicted in Figure 3. GWASpoly uses a Q+K linear mixed model. The model account for population structure and relatedness, and the user can supplement their population structure matrix (Q) of choice. Further details are available in Rosyara et al. (2016).

If QTLs with large effects are discovered, it is possible to design markers that could be used for marker assisted selection. Such markers can be employed early in a breeding cycle to select for a trait before phenotypic data are available. Compared to field and greenhouse trials of all genotypes this could be cost-effective (Slater et al., 2014). A fast and robust way of genotyping a SNP associated with a QTL is the use of kompetetive allele specific polymerase chain reaction (KASP). This technology can score a specific SNP locus. KASP can be used for marker-assisted selection, if the SNP marker and QTL is closely linked and there are little recombination between them (Kante et al., 2021).

Genomic selection is also proposed as a way of accelerating genetic gain in potato and speed up breeding (Slater et al., 2016). Genome-wide SNPs are a prerequisite for this method as well. It can be used to combine many desirable QTLs of small effect, that are difficult to detect, in a new cultivar. In genomic selection a training population is genotyped and phenotyped to produce and validate a model. This model can be used to estimate breeding values of potential parents for a cross, or to select clones by estimating their genetic values (Bradshaw, 2021).

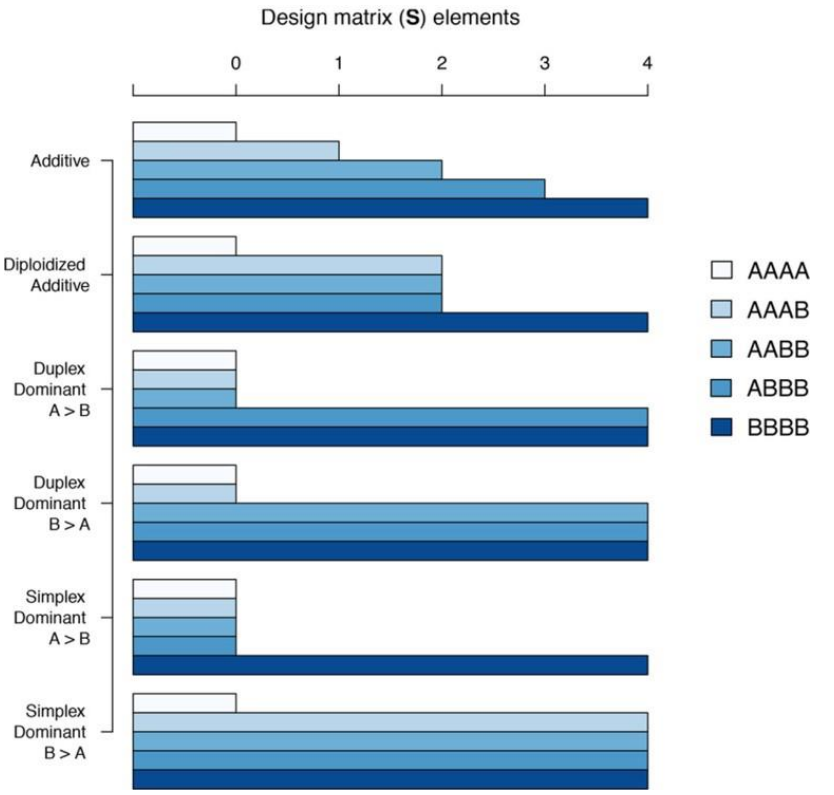

Figure 3. Tetraploid genetic models available in GWASpoly. A and B are alleles of a biallelic single nucleotide polymorphism (SNP). The dominance between alleles is depicted with > and <. Adapted from Rosyara et al. (2016)

1.6. Objectives and overview of the study

The objective of this thesis was to evaluate a cost-effective and high-throughput laboratory method to assess resistance against foliage late blight in potato caused by the pathogen *P. infestans*. It was also an aim to identify and validate molecular markers linked to genes involved in the response to *P. infestans* infection of the foliage in Graminor potato breeding material. To achieve these goals we established an experiment using the "Detached leaf test for foliage blight resistance" (Colon et al., 2004) method with 255 cultivars and breeding material representing the Norwegian germplasm. Plant material was grown in greenhouse and detached leaflets were inoculated and assessed for disease severity. Correlation between the observed resistance reaction in greenhouse-grown plants and historical field resistance data were evaluated. All material was previously genotyped with a SNP marker assay. Genome-wide association study (GWAS) was performed based on the disease severity from detached leaf test and genomic information from the SNP array to identify SNP markers linked to late blight foliage resistance.

2. Materials and methods

2.1. Growth of potato genotypes

2.1.1. Genotype selection

A total of 255 *Solanum tuberosum* cultivars and breeding material (genotypes), representing the Norwegian potato germplasm, were selected for inoculation with *Phytophthora infestans*. All plant material were provided by Graminor AS. The genotypes were split into three batches, with 20 genotypes overlapping in all three batches (Table 1). The 20 genotypes were selected by breeders at Graminor. Five standard cultivars for late blight resistance testing from Eucablight project were included; 'Alpha', 'Bintje', 'Eersteling', 'Gloria' and 'Robijn' (Hansen et al., 2007). Both resistant and susceptible, and early and late genotypes were represented among the overlapping genotypes. Batches 1,2 and 3 consisted of 97, 99 and 99 genotypes, respectively. In addition, four of the overlapping genotypes were also used as negative controls (mock-inoculated with potato water) in every batch (Table 1).

Tubers of all genotypes had been propagated the year before at Graminor AS (approximately, 60°48′N, 11°12′E). Normal agronomic practices regarding fertilization, spraying, irrigation, and haulm topping were followed during propagation. Some tubers were from plants grown in plastic pots in a plastic tunnel. These plants received fertilizer through drip irrigation. The genotypes in tunnel were planted after the propagation field, but all plants were haulm topped at the same time.

Table 1. List of the 20 genotypes used as overlapping control. Maturity is given in brackets, E = early, I = intermediate, L = late. Foliage resistance against late blight is given in parentheses, S = susceptible, M = medium, R = resistant. Genotypes also used as negative controls (mock-inoculated) are marked with an asterisk *.

Alpha [L] (M) *	Carolus[1] [I] (R)	G06-1033 [E] (S)	Mandel 6 [L] (S) *
Arielle [E] (S)	Carolus, new[1] [I] (R) *	Gloria [E] (S)	Nansen [I-L] (R)
Asterix [I-L] (M)	Colomba [E-I] (S)	Hassel [E] (S)	Robijn [I-L] (R)
Athlete [E] (M)	Eersteling [E] (S)	Innovator [I-L] (R)	Sarpo Mira [I-L] (R)
Bintje [E] (S) *	Escort [I] (R)	Labella [E-I] (M)	Saturna [I] (M)

[1] Graminor have two Carolus genotypes in their collection. The first, here named 'Carolus', had abnormal leaves. Therefore, they received a genotype with normal leaves, here 'Carolus, new'.

Table 2. Overview of experiments and timings.

	Week							
	20	24	25	26	32	33	34	35
Batch 1	In pots and cold storage	Placed in greenhouse			Inoculated leaflets	Scored		
Batch 2	In pots and cold storage		Placed in greenhouse			Inoculated leaflets	Scored	
Batch 3	In pots and cold storage			Placed in greenhouse			Inoculated leaflets	Scored
P. infestans	Agar plates prepared	Agar plates inoculated			Used	Used	Used	

2.1.2. Planting of tubers

Tubers were taken out from cold storage 21st of May and potted into plastic pots (3 L, d = 20 cm). All tubers had been stored in paper bags in a cold storage, 4 °C, since harvest. A peat sand mixture (Go' jord, NORGRO, Degernes, Norway) was used as growing medium. One tuber was planted in each pot, apart from genotypes with very small tubers where multiple tubers were planted to ensure sprouting (Appendix 1). Sprouts were kept on seed tubers in batch 1 and removed from seed tubers in batches 2 and 3. After planting, pots were stacked on pallets and covered with plastic to conserve the humidity of the soil, and then stored at 6 °C for 3–5 weeks.

Pots were then taken out from cold storage and placed on greenhouse tables according to the time schedule shown in Table 2. The earliest genotypes emerged above the soil surface during cold storage and had etiolated sprouts (Figure 4). These sprouts quickly de-etiolated after exposure to light in the greenhouse. The pots were placed quite densely on the greenhouse tables due to space limitations. This resulted in plants entangling into each other. The plants were irrigated with tap water by mist sprayer bottles during early development. Later fertilized water was provided by an ebb-flow system with an EC of 1.5. There were no registered attacks of pests or disease in the greenhouse, but beneficial insects against aphids were used as preventive treatment. In the beginning of the experiment the temperature was regulated as 18 °C/ 15 °C day/night. Later when the plants from batch 3 were established, temperature was adjusted to 15 °C/ 10 °C day/night. Lighting relied only on natural conditions. It should be mentioned that the automated climatic control of the greenhouse had problems closing the roof hatches during sudden torrential rain, resulting in extra water for the plants growing in the middle of the greenhouse tables. Technicians at Graminor were responsible for the greenhouse cultivation.

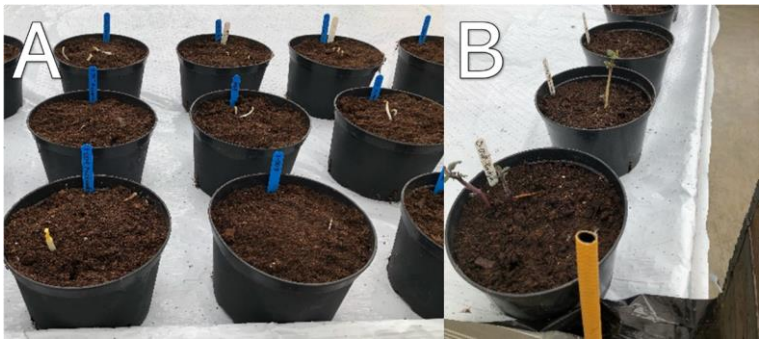

Figure 4. Pots with sprouting potato tubers. **A** shows etiolated sprouts emerging in several pots after cold storage. **B** shows de-etiolated sprouts after one week in the greenhouse.

2.2. Production of *Phytophthora infestans* inoculum

2.2.1. Origin of inoculum

The inoculum was provided by NIBIO in July 2020 and maintained at Graminor on tuber disks of cultivars 'Folva' and 'Laila'. The inoculum provided was a mixture of four different SSR-genotypes of *P. infestans* on detached leaves. The highly virulent isolate EU41_A2 was among the isolates. The first transfer to tuber slices was done by sandwiching in an infected leaflet between two tuber slices of cultivar 'Laila'. The tuber slices were 1 cm thick and cut from a tuber that had been surface sterilized by dipping in 75 % ethanol and flamed off. Subsequent transfers were done weekly by inoculating in 4 points on new tuber slices with mycelium from the old tuber slices. Inoculated tuber slices were placed on a metal grid in a plastic box with a lid. The inoculated side of the slices were oriented downward to allow the pathogen to grow through the slices. After the transfer, the slices were incubated at 15 °C in complete darkness for 1 week until the next transfer. The transfers were performed by Graminor technicians.

2.2.2. Propagation on Rye B Agar

Rye B Agar for sporulation of *P. infestans* was prepared after Caten and Jinks (1968) with the modifications from Cornell University (2013). Three litres of media were prepared as following: 180 g of rye grains were soaked and covered by deionized water in a plastic box. Deionized water throughout the experiment was collected from a Milli-DI Water Purification System (Merck Millipore) with a large DI-PAK Purification pack filter (Merck Millipore) which also removed chlorine, colloids and ionic impurities. The plastic box was covered with aluminium foil and placed in a cupboard for 24 hours at room temperature. The following day

the liquid, 265 ml, was poured off to a beaker and put aside. The grains were transferred to a cooking pot and deionized water was added to 2.5 cm above the grain. The pot was covered with a lid and the rye was boiled for 60 min. After boiling, water and grains were filtered through four layers of cheese cloth into a new beaker. The filtrate was measured to 318 ml. The liquids in the beakers were shaken, measured into equal amounts with a graduated cylinder and transferred to three 1 L glass bottles with screw caps.

To each of the three 1 L glass bottles, 20 g sucrose (Merck Millipore), 15 g Bacto agar (GBiosciences) and 0.05 g β-sitosterol (Merck Millipore) were measured in weighing vessels and added. Deionized water was added to adjust the volume to 1 L in each bottle and followed by autoclaving for 20 min at 121 °C. After autoclavation, bottles were stored in a warming cabinet at 60 °C to prevent solidification. In a laminar flow cabinet, the medium was poured into 9 cm Petri dishes. Agar plates were cooled, wrapped in plastic bags, and placed on a bench in room temperature until use. In total, 146 plates with Rye B Agar were prepared.

Four weeks later the Rye B Agar plates were inoculated with *P. infestans* from potato slices maintained as described earlier. Two of the agar plates were contaminated and discarded. The remaining 144 agar plates were inoculated in the centre with an inoculation loop in a laminar flow cabinet and placed in stacks in plastic bags at 18-20 °C with 8 hours day and 16 hours dark. Lighting was artificial with fluorescent tubes. After 7 weeks, 57 agar plates were discarded due to contamination and the remaining 87 agar plates were placed in 15 °C with 16 hours day and 8 hours dark. Plates were also scattered so that every plate was exposed to light. After 7 days, the temperature was increased to 18-20 °C again, still with 16 hours photoperiod. The inoculation on agar plates was performed by Graminor technicians.

2.2.3. Inoculum preparation

To extend viability of the zoospores, spores were prepared in extracts of potato tubers. Extracts were prepared after McKee (1964). Tubers of cultivar ‘Solist’ were harvested from Graminor’s field, washed and peeled. 300 g of potato was sliced and boiled in 1 L deionized water for 20 min. After the debris settled, the suspension was transferred into three glass bottles. The bottles were autoclaved at 121 °C for 15 min and stored for one to three weeks. The potato tuber extract would later be applied in inoculum preparation to help zoospores remain motile for a longer period of time (Colon et al., 2004).

Figure 5. Preparation of inoculum. **A.** Cell spreader used to release sporangia. **B**. Filling of counting chamber in hemocytometer. The last two are taken through microscope and show sporangia in counting chambers. **C.** Empty sporangium and two non-germinated sporangia. **D**. Two sporangia with large vacuoles.

Inoculum was prepared fresh before harvesting leaves and starting the experiment. To prepare inoculum for a batch, potato tuber extract was diluted to 1 % with autoclaved deionized water in a beaker. Rye B agar plates with mycelium and sporangia were flooded with cold 1 % potato tuber extract. To release the sporangia a glass rod was used in batch 1 and a plastic hockey-stick cell spreader was used for batch 2 and 3 (Figure 5A). To ensure a high concentration of sporangia the same liquid was transferred between plates, to combine the harvest from 4–5 plates. In preparation for inoculum for batch 1, about 20 ml was used on 8 plates resulting in 7 ml with concentrated inoculum. In batch 2 and 3, about 55 ml was used on 16 plates resulting in 20 ml with concentrated inoculum. The concentration of sporangia in the inoculum was determined using a hemocytometer (Neubauer Improved, VWR) under a microscope (Zeiss Standard 14, Germany). Both counting chambers were filled with 10 µl inoculum and sporangia were counted in the four corner squares (Figure 5BCD). In every batch, 100 ml inoculum was prepared in a beaker by diluting with 1 % potato tuber extract. In batch 1, a concentration of 15 000 sporangia/ml was used. In batch 2 and 3, the concentration was increased to 34 000 sporangia/ml. After dilution, the sporangial suspensions were covered with parafilm to prevent contamination and placed in darkness in a refrigerator at 5 °C to promote the release of zoospores from sporangia. Suspensions were brought out from the refrigerator after approximately 5 hours, when leaflets had been prepared for inoculation. Before inoculation, the concentrated inoculum was counted again in the hemocytometer to determine the percentage of sporangia that were empty and had released their zoospores.

2.2.4. Inoculum genotyping

An agar plate with *P. infestans* was sent to The Norwegian Institute for Bioeconomics (NIBIO, Ås) for genotyping. *P. infestans* was genotyped using a 12-plex single sequence repeats (SSRs) assay (Li et al., 2013). SSR genotyping does not reveal information about the pathogenicity, but it can show the diversity in populations of *P. infestans*.

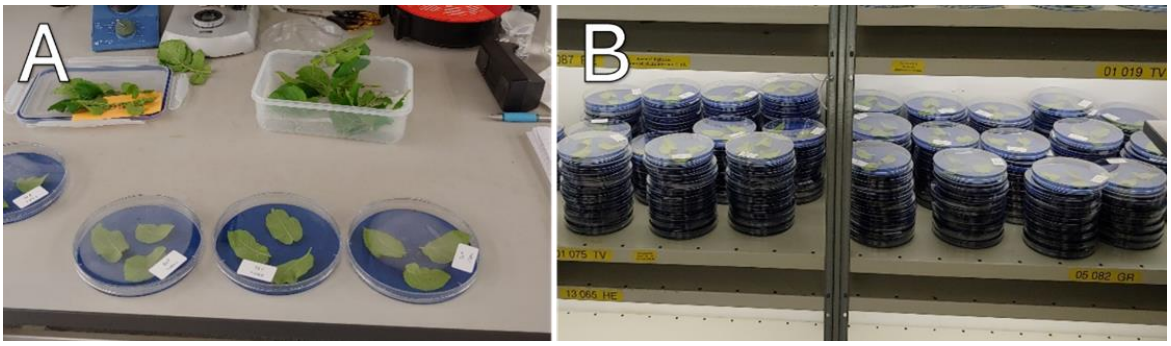

Figure 6. Selection of leaflets before inoculation. **A.** For every genotype 9 leaflets were selected from leaves harvested in the greenhouse and placed in Petri dishes. **B.** Plates with leaflets stacked before inoculation.

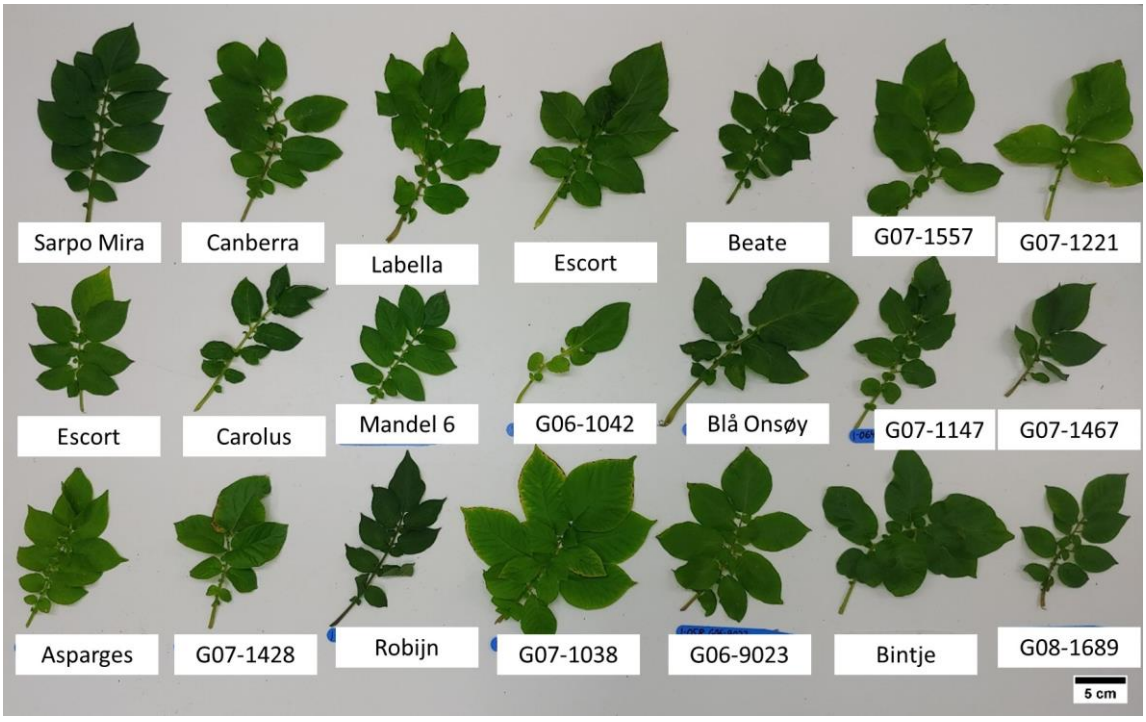

Figure 7. Variation in leaf morphology between potato genotypes. 5 cm bar for scale.

2.3. Inoculation experiment

2.3.1. Harvesting and preparation of leaves

About 7 to 9 compound leaves were collected from every genotype for each batch and placed in plastic boxes with lids. The selected compound leaves were fully developed and located in the upper two-third of the plant. There was an attempt to pick leaves with similar size and age, although variation between the genotypes made it difficult. To prevent desiccation of the leaves, moistened paper towels were placed in the bottom of the boxes. Furthermore, 9 leaflets were picked from the compounds leaves of each genotype and placed into three 14 cm Petri dishes (VWR) (Figure 6). In the Petri dishes, leaflets were placed with the abaxial side upwards on Crocker Blue blotter circles (Anchor Paper Company, Minnesota, USA) that had been evenly moistened with 13 ml deionized water. When selecting leaflets, even sized leaflets were targeted, so that the leaflet area between petri dishes were comparable, and lateral leaflets were targeted before the terminal ones. There were differences in leaf morphology and shape between the genotypes and between the leaves of a genotype depending on their position (Figure 7).

2.3.2. Inoculation of leaves

Leaflets were inoculated using a repetitive pipette, Handystep Touch (BRAND), with Dispenser-Tip PD-Tip II 0.5 ml BIO-CER (BRAND). Multi-dispensing mode was used with an aspiration and dispensing speed of 6 (range: 1 = slow, 8 = fast). One genotype was inoculated at the time. The 3 plates were placed on the counter without lid. 500 µl of inoculum was aspirated in the dispenser tip and a single 50 µl droplet was placed on the abaxial side of the 9 leaflets, between the midrib and an edge (Figure 8). The remaining 50 µl in the dispenser tip was dispensed back to the beaker with inoculum. The beaker with inoculum was swirled regularly to prevent sedimentation of spores. On leaflets from some genotypes the 50 µl droplets just rolled off instantly. In these cases, the leaflets were inoculated with two 25 µl droplets. The negative controls were mock inoculated with 50 µl droplets of 1 % potato tuber extract. Two persons cooperated to ensure efficient inoculation. In batch 1 a vortex was also used for swirling, but in batch 2 and 3 the inoculum was only swirled by hand.

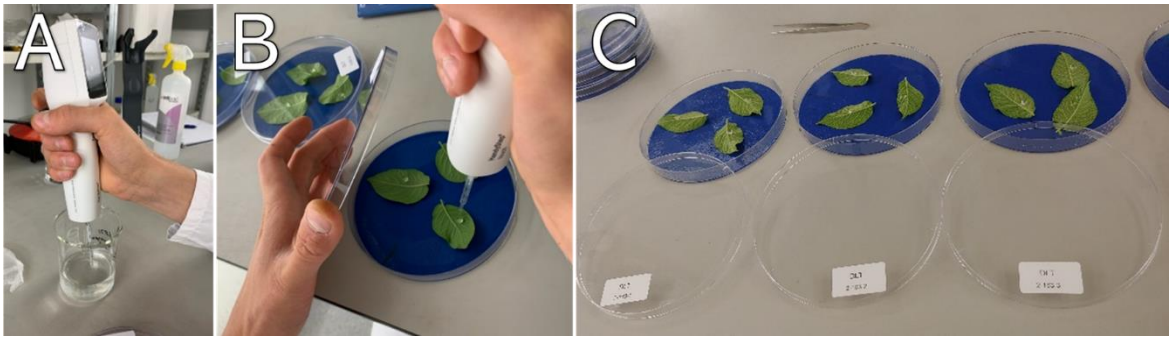

Figure 8. Inoculation of leaflets. **A** shows aspiring inoculum into the pipette. **B** shows placement of droplets. **C** shows all 9 leaflets of a genotype with inoculation drop.

2.3.3. Incubation and growth environment

Leaflets were placed in incubation room at 18 °C and 16 hours photoperiod. Plates were randomized in the incubation room. There were four shelves with two 21 W fluorescents tubes (MININTESA 21W IP40, TCI Srl) placed 37 cm above every height. Illuminance was measured to 3600 lux in the shelves. The fluorescent tubes produced heat that affected the shelves above. Heat from underneath could dry out the leaflets. Bubble wrapping and small fans was placed between the fluorescent tubes and the shelf above for insulation and to circulate the air, respectively. The plates were placed in a brick pattern on the shelves (Figure 9). Measured temperatures ranged between 17 °C and 21 °C.

Leaflets were inspected for presence of inoculation droplets after 24 hours; sufficient time for the pathogen to penetrate the leaflets (Colon et al., 2004).

Following the inoculation, the plant stage of the genotypes were scored after a scale from the Euroblight protocol: 1 = no flower buds visible; 2 = onset of flowering; 3 = flowering and lower leaves yellowing (Colon et al., 2004).

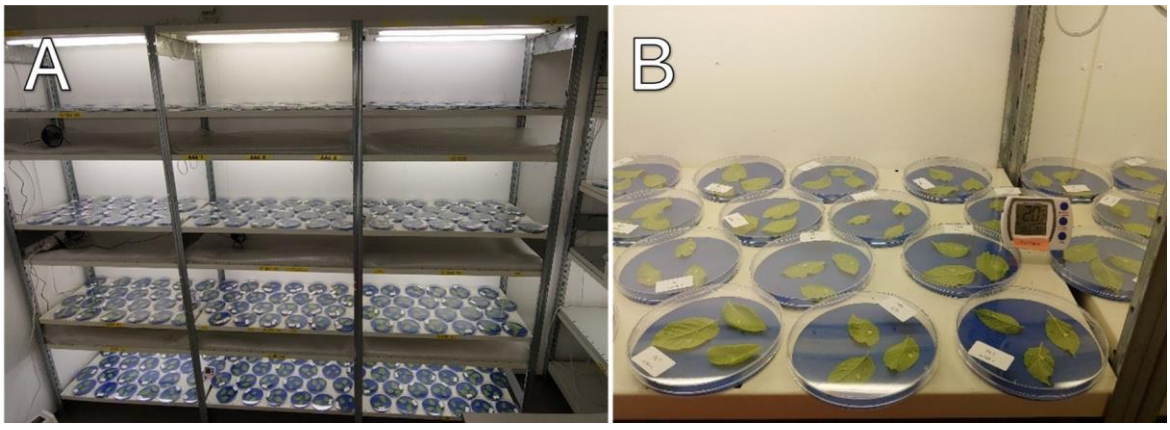

Figure 9. Incubation of inoculated leaflets. **A**. Shelves with inoculated leaflets. **B.** Closeup of the leaflets in the shelves.

2.4. Assessment of disease development

Leaflets were assessed for disease development by eye and through manual image analysis. The time of scoring differed between genotypes and batches, but all were scored visually after 6 days and through image analysis after 6 and 8 days after inoculation. Additionally, batch 1 was scored visually after 8 days, the 20 overlapping genotypes in batch 2 were scored daily from day 1 to day 8 in ImageJ, and some genotypes in batch 3 were scored in ImageJ after 9 days. To limit the exposure to another environment, only 50 plates with leaflets were scored at the same time. In some of the plates the blotter circles showed a lighter shading of blue indicating dryness (Figure 10). The lighter shading was likely due to variation in the paper quality. As an even blue colour on the blotter circles would make later image analysis easier, deionized water was used to wet the blotter papers with lighter shading.

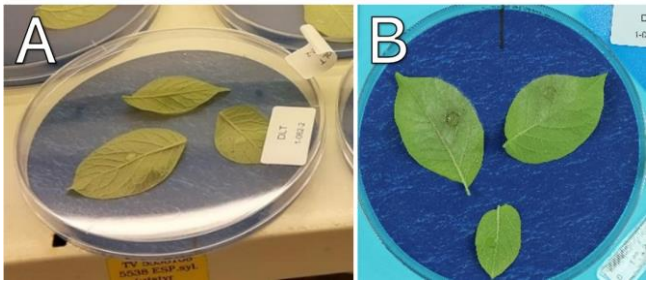

Figure 10. Blotter circles with lighter shading indicating dryness. Viewed in the shelf, **A**, and in the image for image analysis, **B**.

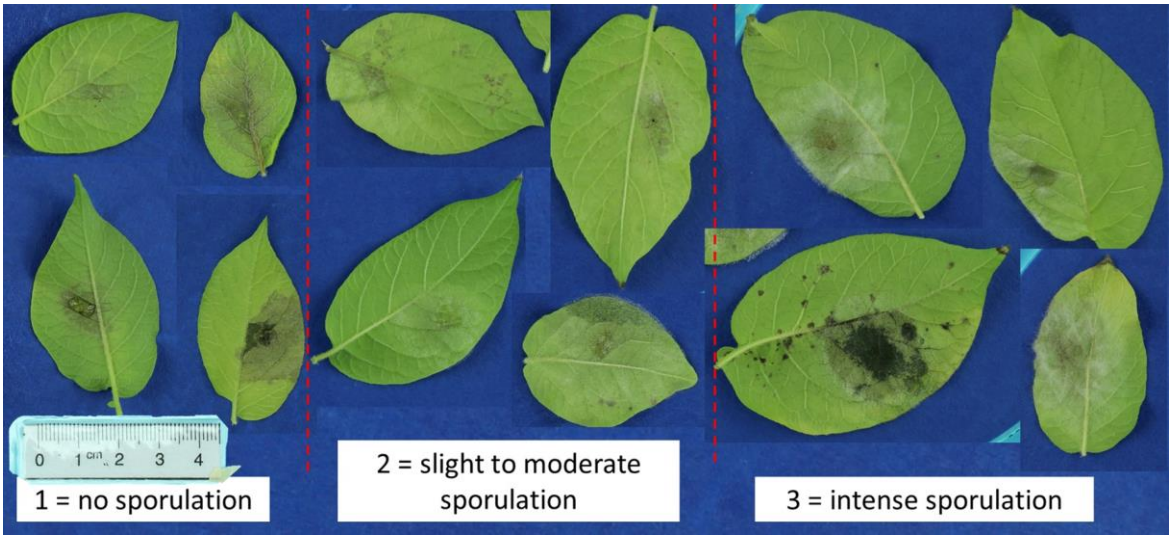

Figure 11. Scale for scoring sporulation of *Phytophthora infestans* on leaflets.

Leaf area affected by the pathogen and the intensity of sporulation were scored visually as described by Colon et al. (2004). Sporulation was scored on a 1–3 scale: 1 = no sporulation; 2 = slight to moderate sporulation; 3 = intense sporulation (Figure 11, Appendix 2). Leaf area was scored in percentage: 0 % = no apparent infection; 0.1 % = small, separate necrotic lesions; 1 % = inoculated area necrotic; 5–100 % = percentage of leaflet infected (Figure 12, Appendix 2). There was a slight adjustment during the scoring of batch 1 in day 6. At first only the area with necrosis or heavily covered with mycelium was scored, but later this was changed so that also area lightly covered with mycelium was scored as infected.

Images for image analysis were taken with a digital camera. Each plate was photographed separately with a ruler for scale, a colour card, and a sample tag (Figure 13B). To provide even lightning two soft-boxes with 85 W bulbs were placed on the sides. Both the bulbs in the soft-boxes and the fluorescent tubes in the room had a light spectrum imitating natural daylight. The camera was mounted on a Hama Repro stand (Hama, Germany). The camera, EOS 1300D (Canon, Taiwan), was equipped with a EFS 18–55 mm lens (Canon, Japan) and operated through the program EOS utility 3 from Canon on a computer (Figure 13A). Remote operation of the camera through a computer allowed steady shots. The following camera settings were used: Aperture priority mode, ISO 100, white balance 5200 K daylight, One Shot focus, Centre-Weighted average metering, Aperture F9. The shutter speed was automatically selected by the camera itself, usually to either 1/15 or 1/20 seconds.

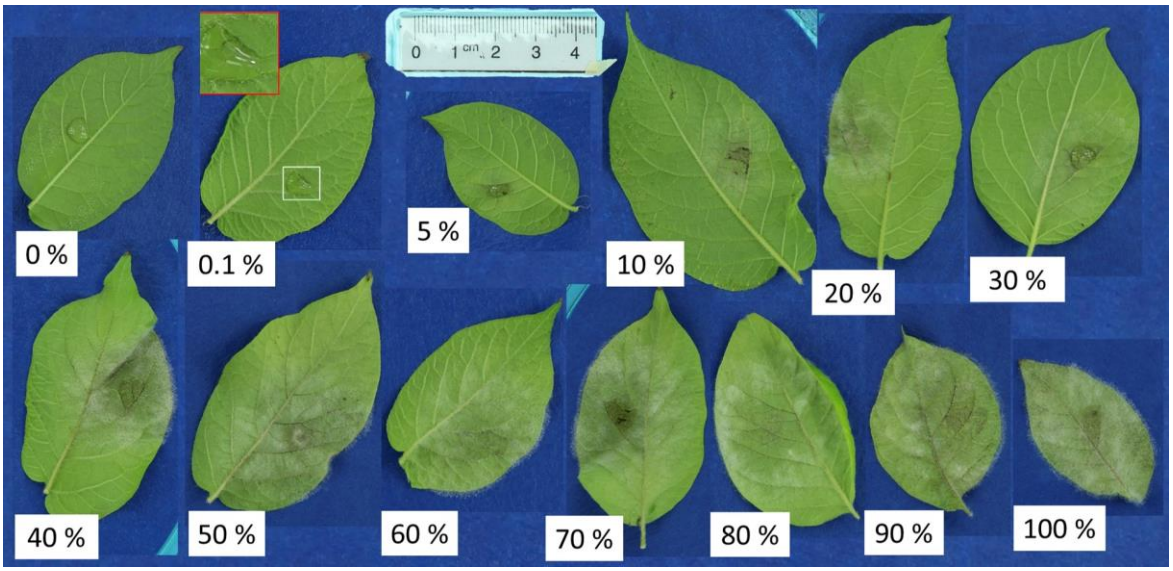

Figure 12. Scale for scoring infected leaflet area, 0–100%.

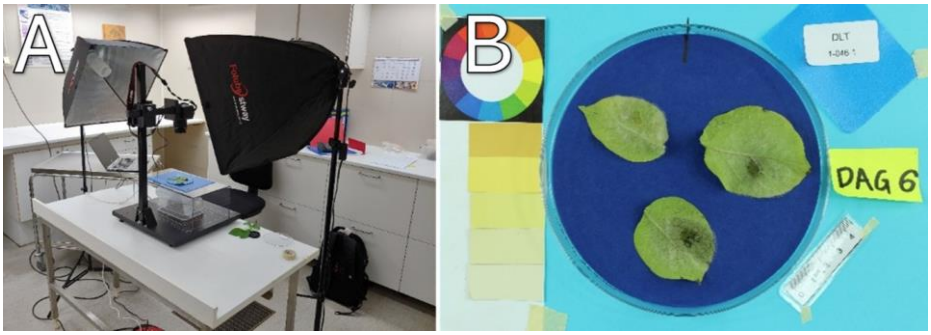

Figure 13. Photography of leaflets on plates. **A**. Setup with softboxes and camera stand. **B**. Example of an image used in image analysis. The colour card, ruler and sample tag were placed around the leaflets in the Petri plate.

2.5. Data processing

2.5.1. Assessing leaflets in ImageJ

All taken images from the infected leaflets were renamed in the Advanced Renamer version 3.88 application (Jensen, 2021) as: batch – genotype no. – plate no. – day of scoring (#-###-#_day##).

Percentage infected leaf area was calculated from the images using the program ImageJ version 1.53K (Schneider et al., 2012) (Figure 14). This allowed a precise quantification of the diseased area that was verifiable and could be repeated. An internal protocol for ImageJ was adjusted to allow more efficient workflow. Every image was imported to ImageJ and scaled with the *Set scale* function after the ruler on the pictures. Afterwards the plugin Threshold Colour v1.16 from Landini (2008), a modification of BandPass2 Filter created by Bob Dougherty's, was used to filter away the blue colours by setting the hue to 0–144. This helped to remove the blue blotter circles from the image, providing a clear outline of the leaflets. By applying a threshold, making the leaflets black on a white background, the leaflets were selected and marked as regions of interest with the tracing tool in ImageJ. The threshold was reverted so that all colours except blue returned, and the diseased area of the leaflets was traced by hand using the freehand selection tool and marked as regions of interest. The measure function in ImageJ was used to calculate the area of the regions of interest. These values were pasted into an Excel spreadsheet and % infected leaf area was calculated using the formula: infected area / total leaflet area.

Leaflets were scored in groups after batches and the day pictures were taken. The latest images were scored first as they had the clearest symptoms, followed by earlier days images.

Figure 14. Manual image analysis in ImageJ. **A**. Image of leaflets on plate. **B**. The blue blotter paper was filtered away using the macro Treshold Color. **C**. Thresholds were applied to outline and select the leaflets. **D**. Infected leaflet area were traced manually by cursor, allowing the program to calculate percentage of infected area for every leaflet.

2.5.2. Data analysis

Data analysis was performed in the software R version 4.0.4 (R Core Team, 2021) with the Tidyverse package version 1.3.1 (Wickham et al., 2019) and in Minitab statistical program (Minitab, 2021). Image series of leaflets to display daily development were made using ImageJ version 1.53K (Schneider et al., 2012). Zoomed insets were created with the "Zoom in Images and Stacks macro tool" (Carpentier, 2010).

The data from the leaflet assessments were converted to 1-9 resistance score using two different methods. The first method uses infected area scored in ImageJ 6 and 8 days after inoculation to calculate Area Under the Lesion Expansion Curve (AULEC) by the formula:

$$\frac{Day\ 3\ \% + Day\ 6\ \%}{2} * 3 + \frac{Day\ 6\ \% + Day\ 8\ \%}{2} * 2, \qquad where\ Day\ 3\ \% = 0$$

The second method used was to multiply infected area scored in ImageJ and sporulation score (1,2 or 3) from day 6. In some genotypes few leaflets got infected. As an attempt to circumvent the lack of infection in leaflets, scores using only the 1 or 2 most infected leaflets in each plate were also calculated, but these calculations gave similar rankings and were not used (not shown).

For all the methods, method scores were converted to a 1-9 scale using the formula:

$$Score = 9 + Method\ score * \frac{1 - 9}{Highest\ method\ score - lowest\ method\ score},$$

where the highest and lowest method scores are the averages of leaflets in the plate with most and least infection.

A statistical analysis was performed using General Linear Model in Minitab. The response variable was the 1–9 scores calculated with the AULEC formula of day 6 and 8 assessment with all leaflets and batch and genotypes were used as random effects, $\text{Score} \sim \text{Genotype} + \text{Batch}$. Leaflets that could not be scored were coded as asterixis, *, and ignored in the analysis. Analysis of variance was run on the model.

Inoculation scores from the detached leaflet assay were compared to Graminor's historical field data from 2004 to 2021. Spearman correlation and Pearson correlation between the field scores and the leaflet assay scores were calculated in R, along with a scatter plot showing the linear relationship.

A genome-wide association study (GWAS) was performed using the R package GWASpoly version 2.09 (Rosyara et al., 2016). Genomic information of 251 of the genotypes were available as they had previously been genotyped with a Illumina 22K SNP potato GGPv3.0 array (Vos et al., 2015). The SNP data were filtered using a custom script down to 11 826 markers.

The population structure of the genotypes was analysed in Structure (Pritchard et al., 2000). The genotypes seemed to divide into 2 subpopulations (k = 2) and a Q matrix with the genotypes relatedness to each population was made. Collection of DNA for genotyping, filtering of SNP data and analysis in Structure were performed by employees at Graminor.

The two resistance score calculations based on the inoculation assessment as described above were used as phenotypic data in the GWAS model. Batch was used as a fixed effect in the model, along with the Q matrix. In addition, a maximum genotype frequency (after applying dominance relations) of $1 - \frac{5}{N}$, where N = the number of genotypes, was used as recommended in the GWASpoly vignette. The K-model (Yu et al., 2006), which are used in GWASpoly to control for population structure, was calculated with the leave-one-chromosome-out (LOCO) method where only markers from other chromosomes are used to calculate the covariance matrix (Yang et al., 2014). The analysis was run on the gene action models additive, simplex dominant, duplex dominant, and general. Quantile-quantile plots were used to check inflation of the -log10 (p) values. If the values deviate heavily from the dotted line the model assumptions may be broken. A significance level of 0.05 was set as discovery threshold, using the M_{eff} method for correction of multiple comparisons (Moskvina & Schmidt, 2008). The results were visualized using Manhattan plots and significant markers were extracted. GWAS was also performed only using data of from the three batches independently, instead of all together, but without the Q matrix as a fixed effect.

Principial component analysis (PCA) was run on the 255 genotypes and compared to the Q matrix from Structure. GWASpoly was used to convert the SNP genotype data from a nucleotide format to a numeric format. Nucleotide bases were used as reference alleles in order A > C > G > T and the numerical genotype was the same as the number of copies of the reference allele, meaning that a genotype with AAAT would be converted to 1, while CGGG would be converted to 3. The principial component analysis were performed with the prcomp() function in R.

3. Results

3.1. Growth of genotypes

All tubers sprouted and produced healthy plants that grew for 8 weeks before the leaves were harvested. For two of the genotypes in batch 3, 'Hassel' and 'Mozart', only a single stem grew from the tuber. However, the single stems provided sufficient leaves for the inoculation experiment, so the genotypes did not have to be dropped.

The majority, 252 of 295 plants were scored to plant stage 2, onset of flowering, and 31 plants were scored to plant stage 3 as they had finished flowering while the lower leaves had begun to yellow. No flower buds were visible in 10 of the plants, plant stage 1. The distribution of plant stages between batches were quite even (Table 3). Batch 2 had more plants in stage 1 and 3 than the others, but when only comparing the overlapping genotypes batch 3 had more plants in plant stage 3.

3.2. Detached leaflet assay

3.2.1. Discarded and contaminated leaflets

It was challenging to score infection for some of the leaflets. If the droplet with inoculum was not observed after 24 hours and no infection occurred, the leaflet was discarded. Leaflets that were infected by other pathogens or severely damaged were also discarded. The total number of the discarded leaflets were 56. Both negative controls and inoculated leaflets were contaminated by other pathogens (Figure 15). Five genotypes, 'Van Gogh', 'G07-1151', 'Peik', 'N-86-10-32' and 'Tromøypotet', had four out of nine leaflets that were discarded. These genotypes were, therefore, excluded from later data analysis.

Table 3. Potato plant stage at the time of leaf collection for inoculation. 1 = no flower buds visible; 2 = onset of flowering; 3 = flowering and lower leaves yellowing. The scores for only the 20 overlapping control genotypes are in parentheses.

Batch*	**Plant stage**		
	1	**2**	**3**
1	2	88 (18)	7 (2)
2	6 (1)	79 (17)	14 (2)
3	2	85 (16)	12 (4)

* The 255 genotypes were divided into 3 batches, with 20 overlapping genotypes present in every batch.

Minor contamination could also be observed on some leaflets (Figure 16). Furthermore, it was difficult to distinguish early symptoms of *P. infestans* due to black discoloration (Figure 16). The contamination covered up any eventual necrotic lesions. First assessing the oldest picture of a leaflet, from day 8 or day 9, was helpful to determine if there were any *P. infestans* infection. Leaflets actually infected by *P. infestans* developed visible symptoms at those days, while leaflets only contaminated did now show such progression. Leaflets with minor contamination were not discarded.

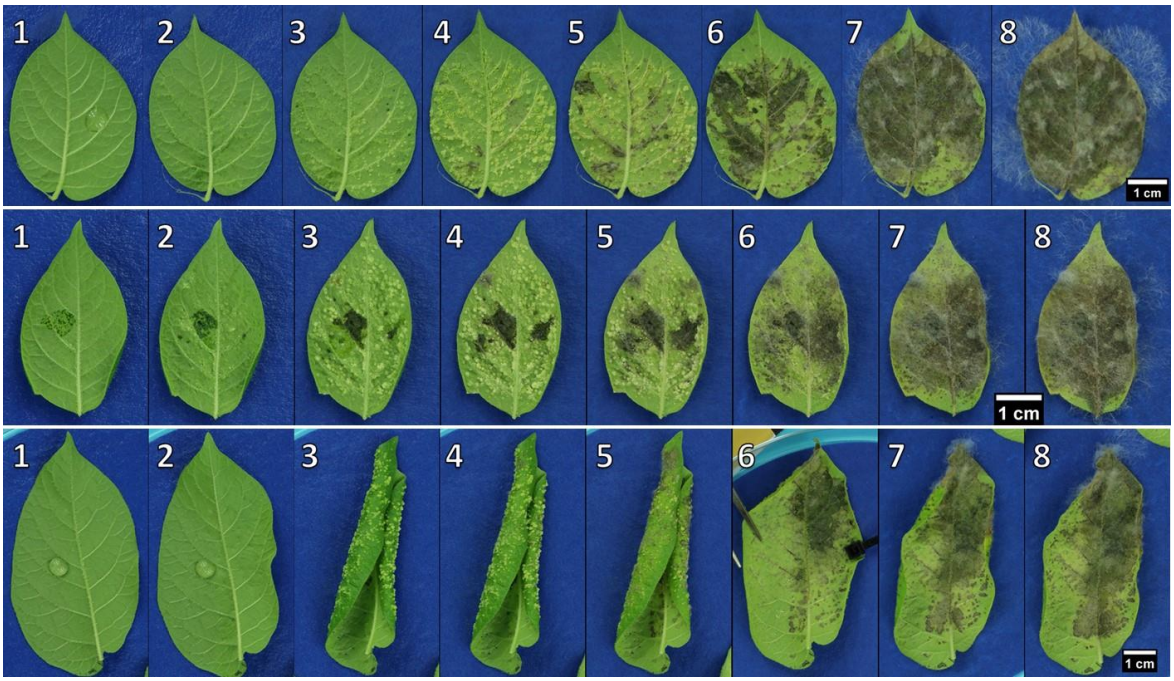

Figure 15. Examples of leaflets with contaminating infections. 1–8 days after inoculation with *Phytophthora infestans* or mock. Top panel: Escort. Middle panel: Carolus ny, negative control (inoculated with potato 1 % tuber extract). Bottom panel: Bintje, negative control. 1cm bar for scale.

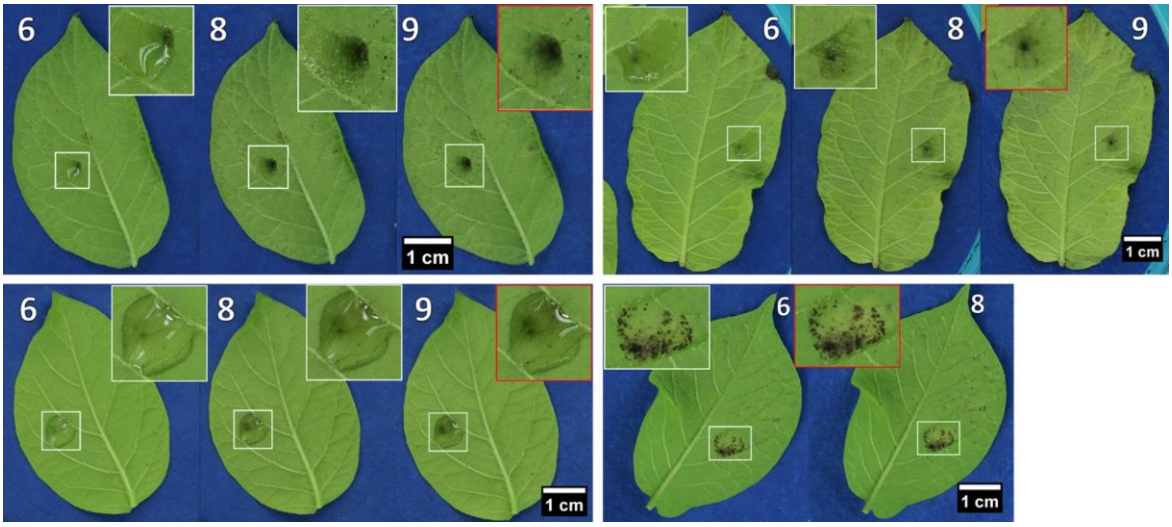

Figure 16. Examples of leaflets with minor contamination around the inoculation droplet. 6–9 days after inoculation with *Phytophthora infestans*. Top left panel: Carolus. Top right panel: Liva. Bottom left panel: N-92-15-138. Bottom right panel: G11-4027. 1 cm bar for scale.

3.2.2. Incidence

Many inoculated leaflets did not show any signs or symptoms of *P. infestans* infection. In addition, infections varied between leaflets of the same genotypes in the same plate; e.g., two healthy leaflets and one severely infected leaflet or the opposite were observed. In some cases, necrotic lesions were not observed under or around the drop (Figure 17).

Typical symptoms of *P. infestans* were visible on 1–3 leaflets of 138 genotypes, and on 4 or more leaflets of 95 genotypes (Figure 18). In 57 genotypes, no symptoms were visible on any leaflets. Overlapping genotypes are counted three times (once for each batch). The threshold of symptoms was set to 1 % leaflet area infected 8 days after inoculation, as contamination made assessments below this uncertain.

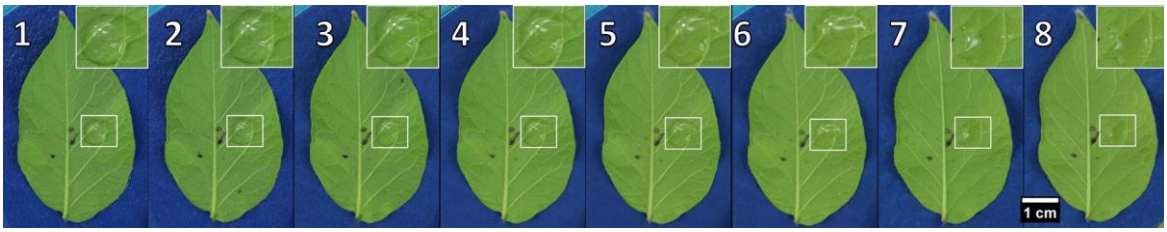

Figure 17. A leaflet of Colomba seemingly unaffected by the inoculation droplet 1–8 days after inoculation with *P. infestans*. 1 cm bar for scale.

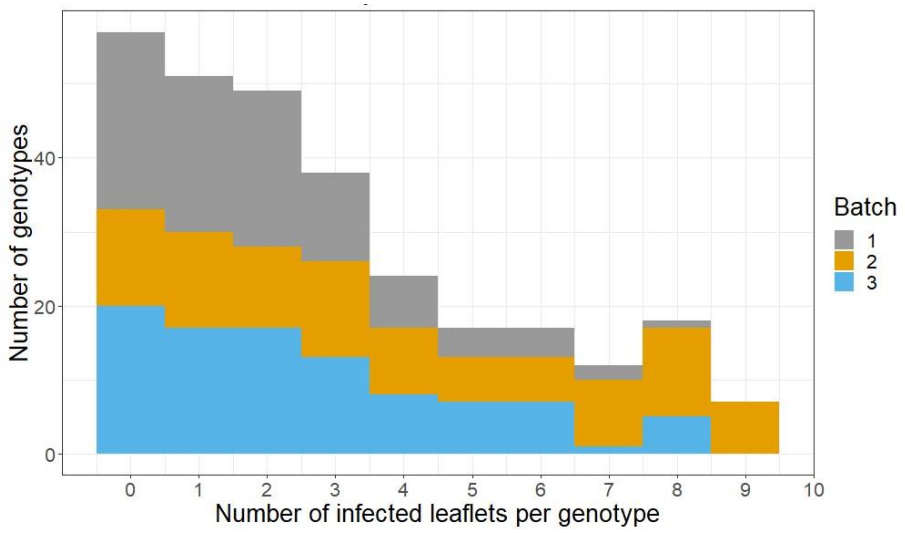

Figure 18: Distribution of infected leaflets per genotype. Overlapping genotypes are counted three times. Leaflets with higher than 1 % area infected 8 days after inoculation assessed in ImageJ are counted as infected. Bars are coloured after batches.

3.2.3. Visual versus image assessment

In this study, visual assessment by eye and assessment by image analysis showed correlation. Linear regression analysis comparing the two methods was performed on leaflets with ≥ 1 % infected area in both methods. With the exception of leaflets from batch 1 day 6 that were assessed before scoring adjustment, the predicted correlation was high, with R^2 values ranging from 0.89 to 0.96 (Figure 19). The regression slopes for day 8 in batch 1 and day 6 in batch 2 and 3 ranged from 1.00 to 1.10. In batch 2 and 3, there was a tendency that leaflets with < 40 % area infected in ImageJ were scored lower in the visual assessment, while leaflets with 40–70 % area infected in ImageJ were scored higher in the visual assessment.

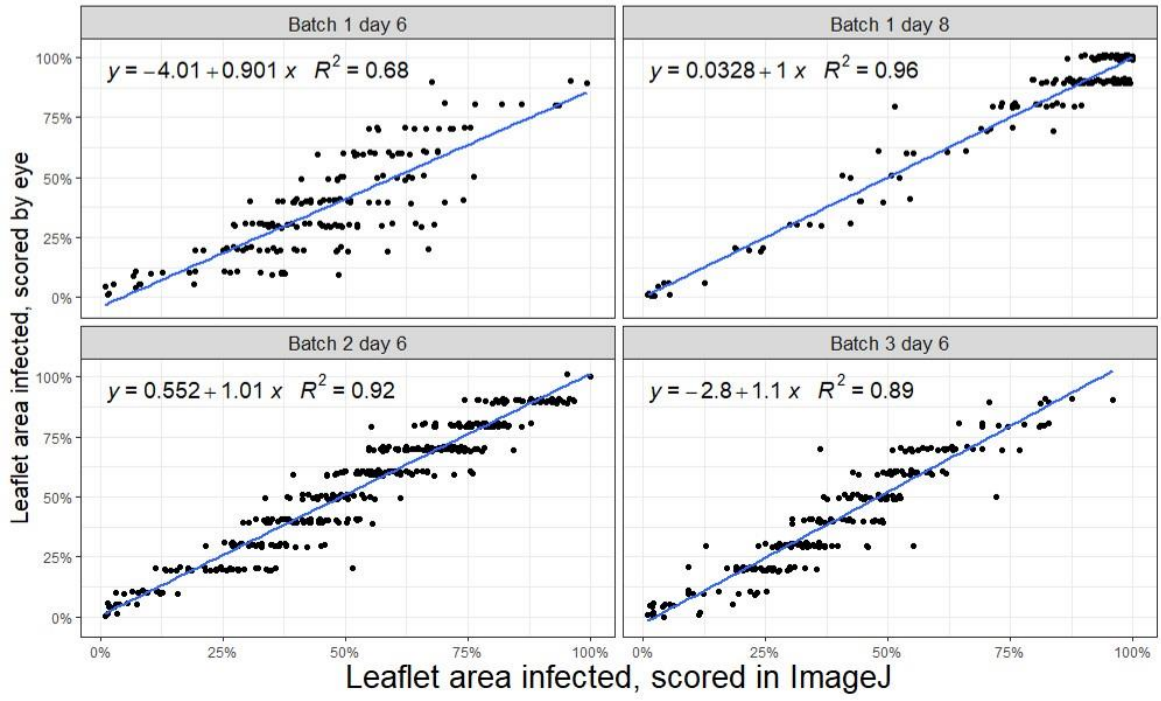

Figure 19. Comparison of scoring in ImageJ and by eye. Leaflets were scored by eye and photographed for later scoring in ImageJ. The figure shows the relationship between these two ways of scoring and a regression line with correlation. Leaflets were scored by eye twice in batch 1 and once in batch 2 and 3. There was an adjustment to the method of scoring by eye during batch 1 day 6.

3.2.4. Day of scoring

All batches of leaflets were scored using ImageJ 6 days and 8 days after inoculation. When comparing leaflets with > 1 % infected area from all batches, there was a clear tendency that scores were more evenly distributed at day 6 than day 8 (Figure 20). At day 8, the scale was more saturated as most leaflets scored closer to 100 %; therefore, further scoring could not detect differences between genotypes. The same pattern can also be seen when only comparing overlapping genotypes between the batches (Figure 20). Genotypes in batch 2, that were scored daily, gave a good insight on the disease progression in the leaflets (Figure 21 and Figure 22). There were differences between genotypes in when the first symptoms appear, but all leaflets featured a characteristic stage of linear growth that flattened out as the infected area approached 100 %. There were examples of leaflets that had very minor symptoms 6 days after inoculation but showed clear symptoms 8 days after inoculation (Figure 23).

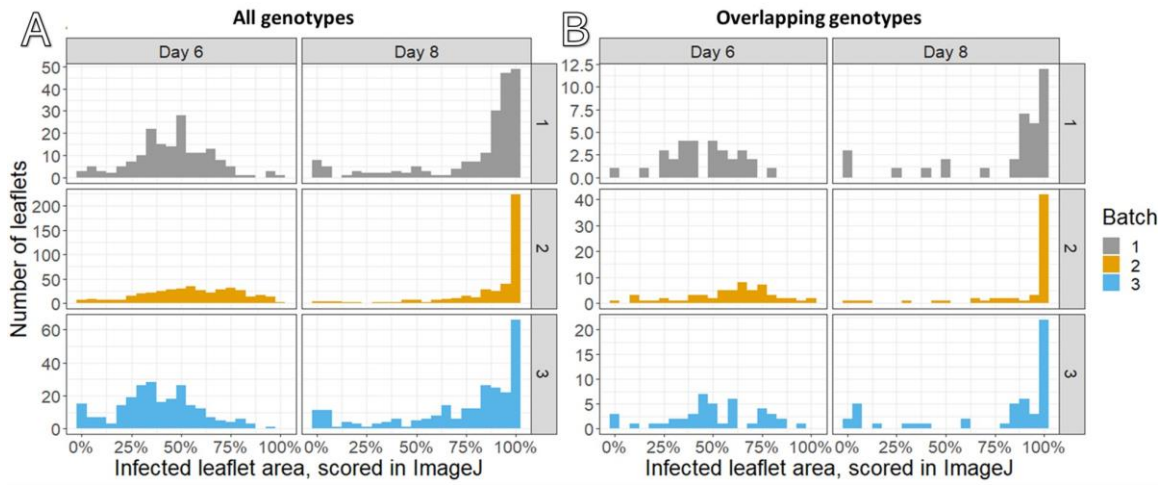

Figure 20. Distribution of % infected leaflet area assessed in ImageJ 6 and 8 days after inoculation with *Phytophthora infestans*. Only leaflets with > 1 % infected area are shown. Data are grouped after batch and Y-axis is different between batches. **A**. Leaflets of all genotypes **B.** Only leaflets of 20 overlapping

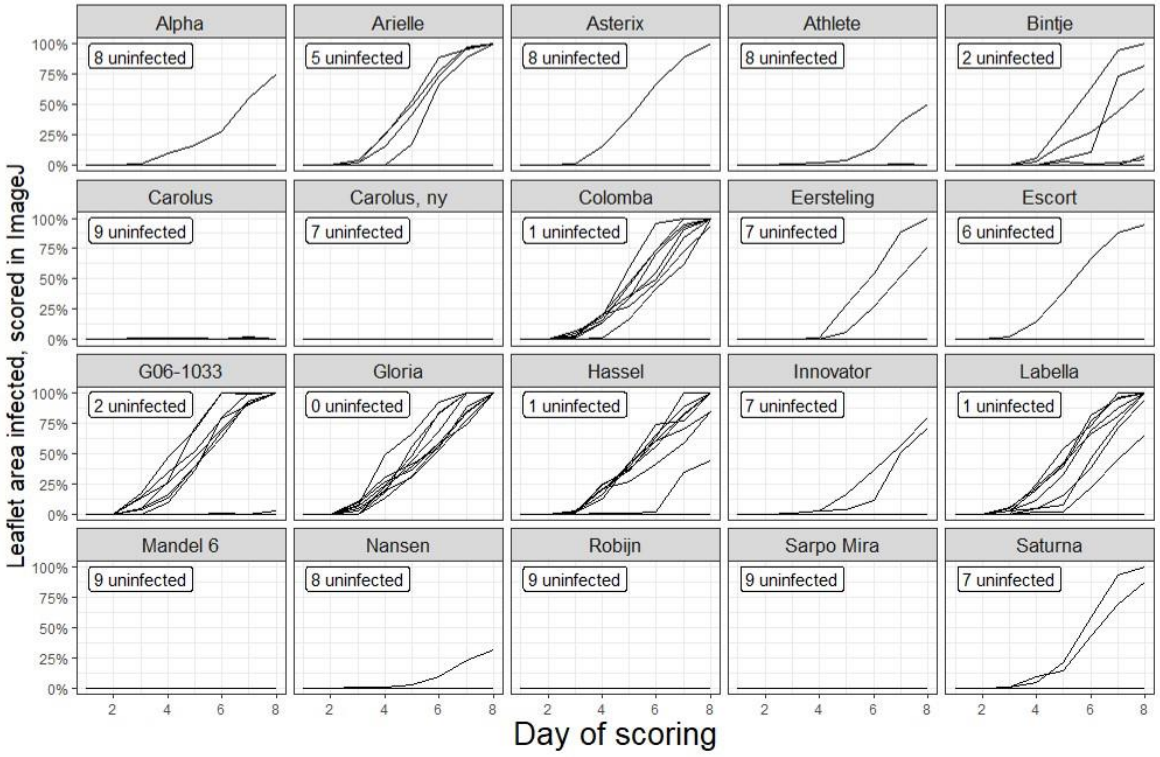

Figure 21. Development of % infected area in leaflets of the overlapping genotypes in batch 2 from 1 to 8 days after inoculation with *Phytophthora infestans*. The number of symptomless/ uninfected leaflets for each genotype are given in the plot.

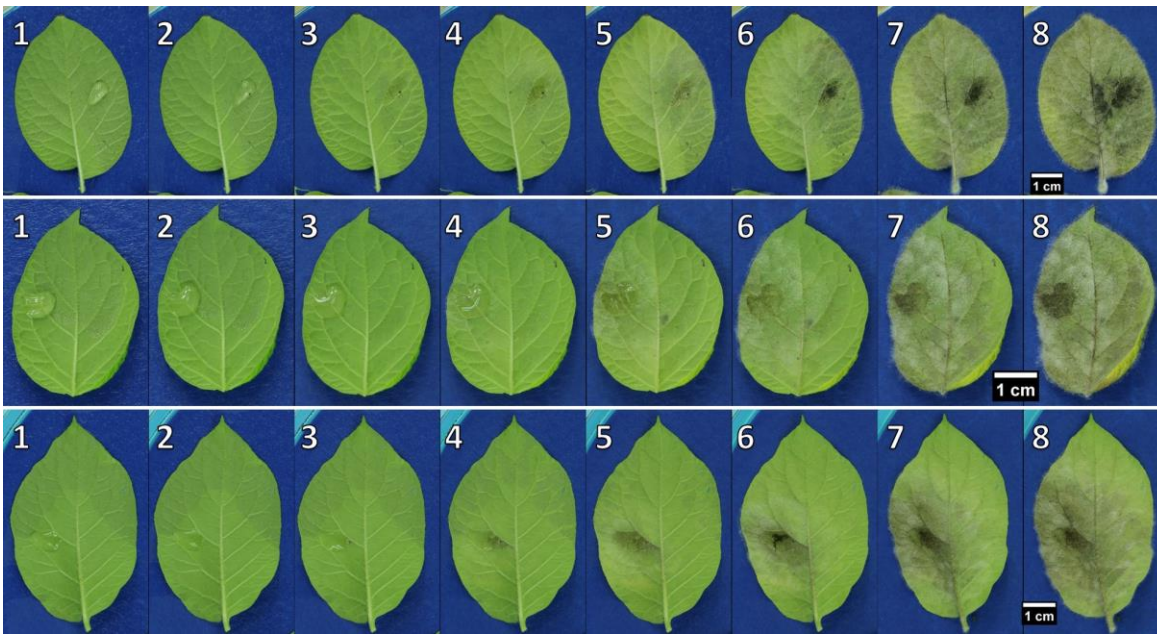

Figure 22. Disease progression in leaflets from 1 to 8 days after inoculation with *Phytophthora infestans*. Top panel: 'G06-1033'. Middle panel: 'Gloria'. Bottom panel: 'Innovator'. 1 cm bar for scale.

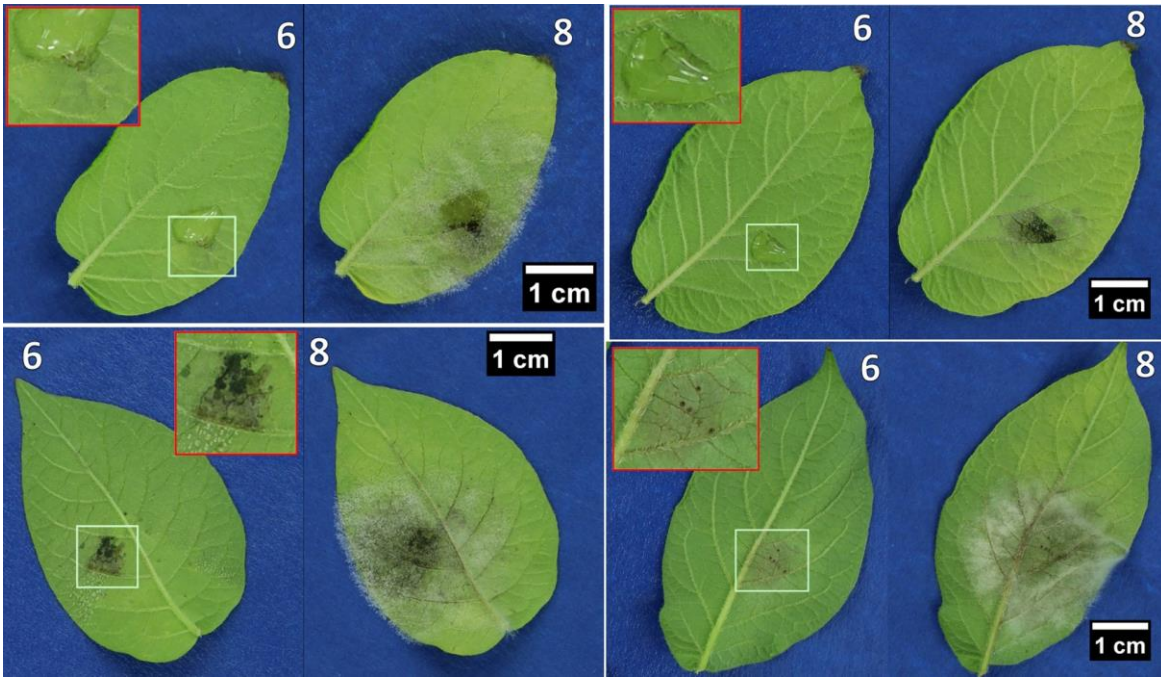

Figure 23. Leaflets with large increase in infected leaflet area between 6 and 8 days after inoculation with *Phytophthora infestans.* Top left panel: 'G06-1422'. Top right panel: 'G10-2066'. Bottom left panel: 'Fontane'. Bottom right panel: 'Gladiator' (2-149). 1 cm bars for scale.

3.2.5. Differences between batches

Although 20 genotypes overlapped between the three batches, it was difficult to determine the consistency in the experiment between the batches. A lower concentration of sporangia (15 000 sporangia/ml) was used to prepare to suspension in batch 1, than batches 2 and 3 (34 000 sporangia/ml). The overlapping genotypes; 'Alpha', 'Athlete', 'Carolus', 'Carolus ny',' 'Mandel 6', 'Nansen', 'Robijn' and 'Sarpo Mira'; had leaflets with little to no infection in every batch (Figure 24). The genotypes; 'Arielle', 'Colomba', 'G06-1033', 'Gloria', 'Hassel' and 'Labella'; had infected leaflets in all batches. While the genotypes; 'Bintje', 'Eersteling' and 'Saturna'; performed inconsistent between the batches.

In batch 1,2 and 3; 28, 53, and 37 leaflets of the overlapping genotypes were infected on 70 % or more of the leaf area 8 days after inoculation, respectively (Figure 20). The same pattern of more infected leaflets in batch 2 applies also when looking at all genotypes (Figure 20).

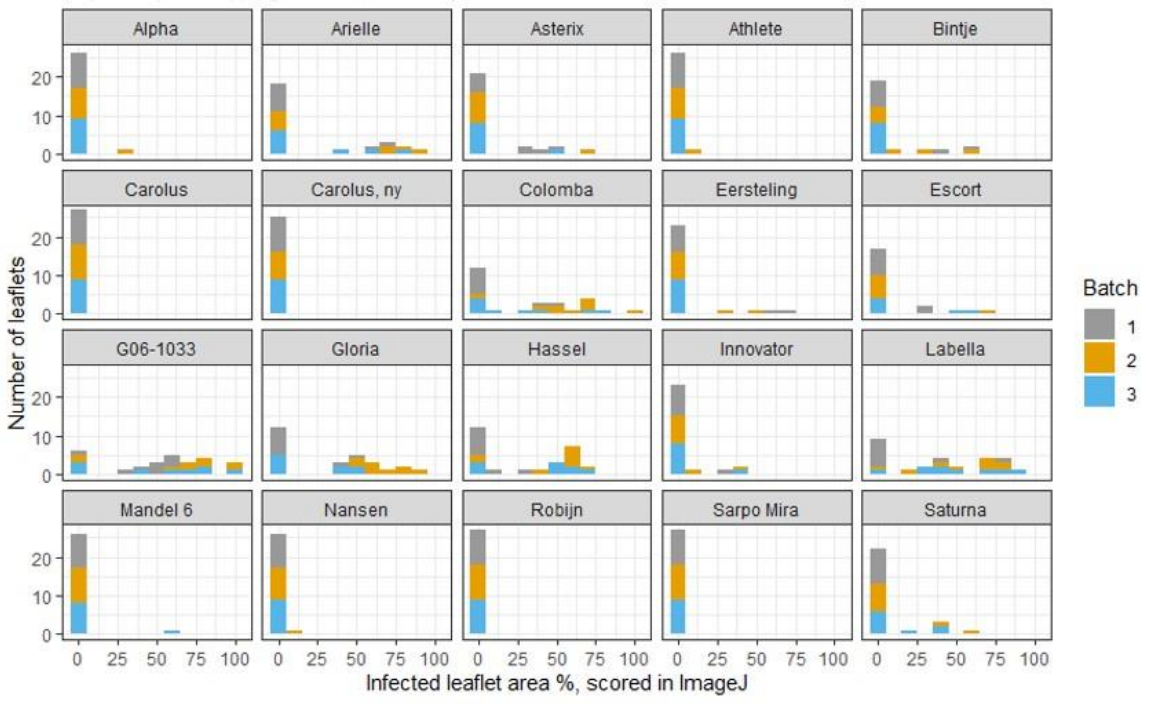

Figure 24. Comparison of the 20 overlapping genotypes between batches. Infected leaflet area assessed 6 days after inoculation in ImageJ.

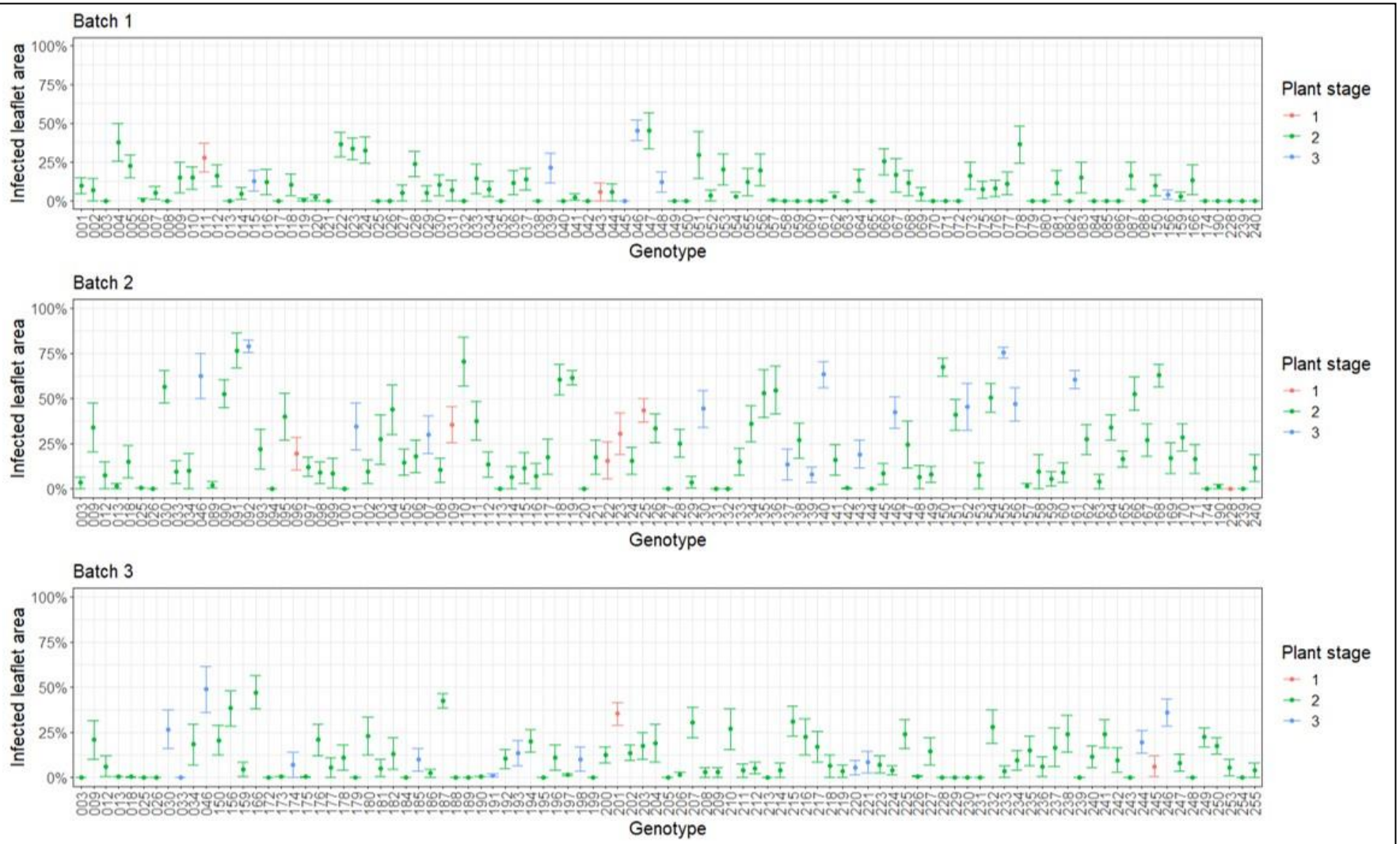

Figure 25. Average infected leaflet area of genotypes 6 days after inoculation, assessed in ImageJ. Whiskers show the calculated standard error. Genotypes are arranged by batch and overlapping genotypes are represented in every batch.

3.2.6. Genotype scores

The infected leaflet area means of the genotypes, scored in ImageJ 6 days after inoculation, show noticeable differences between genotypes (Figure 25). Genotype and batch were significant factors that affected the score (Table 4). The model, Score ~ Genotype + Batch, had a R^2_{adj} value of 33.0 %. The low proportion of infected leaflets resulted in high variance of the genotype score and made it difficult to statistically differentiate genotypes in any post-hoc test (not shown).

The distribution of resistance scores was similar for both methods used to convert the assessments into a 1–9 resistance score (Figure 26). Many genotypes received a high resistance score, giving a left-skewed distribution.

Table 4. Analysis of variance (ANOVA) table from Minitab of the general linear model, Score ~ Genotype + Batch.

Analysis of Variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Potato genotype	254	7388.9	29.09	5.52	0
Batch	2	75.3	37.64	7.14	0.001
Error	2342	12350.8	5.274		
Lack-of-Fit	38	417.3	10.983	2.12	0
Pure Error	2304	11933.4	5.179		
Total	2598	20434.9			

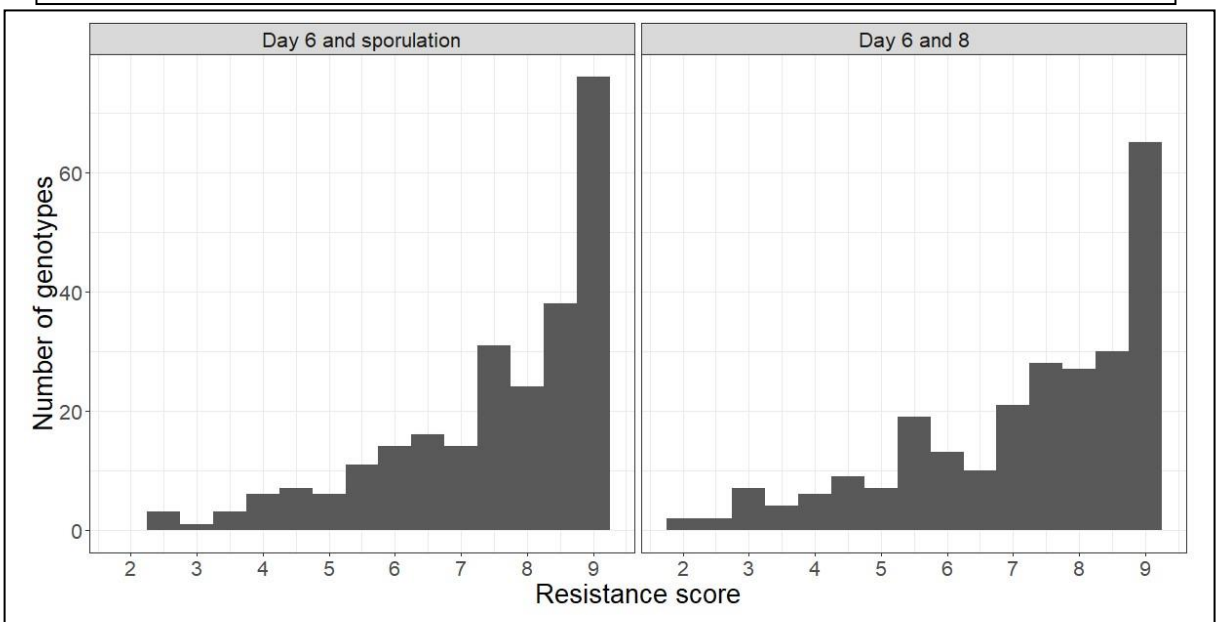

Figure 26. Distribution of resistance scores of 251 genotypes from a detached leaflet assay. Two methods were used for converting assessments to a 1–9 scale, see Materials and Methods. 1= susceptible, 9 = resistant.

3.2.7. Comparison to field scores

Historical field resistance scores were available for 152 of the genotypes used in the detached leaflet assay (DLA) in this research. The distribution of the resistance scores from the DLA of these 152 genotypes were similar to that of all 251 genotypes (Figure 27). In contrast, the resistance scores from breeding field trials were more evenly distributed with the majority of genotypes scored less than 5.5 (Figure 27).

A linear relation and correlation were found between the resistance score of all genotypes from field tests and DLA, Pearson's r = 0.32, p = 0.000 (Table 5). Similar correlations were found evaluating both the linear (Pearson) and monotonic (Spearman) relationship. The differences in correlation between the two DLA calculations were low. Calculating correlation separately for the three batches resulted in higher correlations in batch 2 and 3 (r = 0.41–0.43), but not in batch 1 (r = 0.33–0.35). Attempts at filtering away genotypes with high DLA scores did not improve correlation (not shown). High dispersions were found for individual genotypes (Figure 28). Genotypes were found with a low score in the DLA and a relative high score in the field tests, and the other way around.

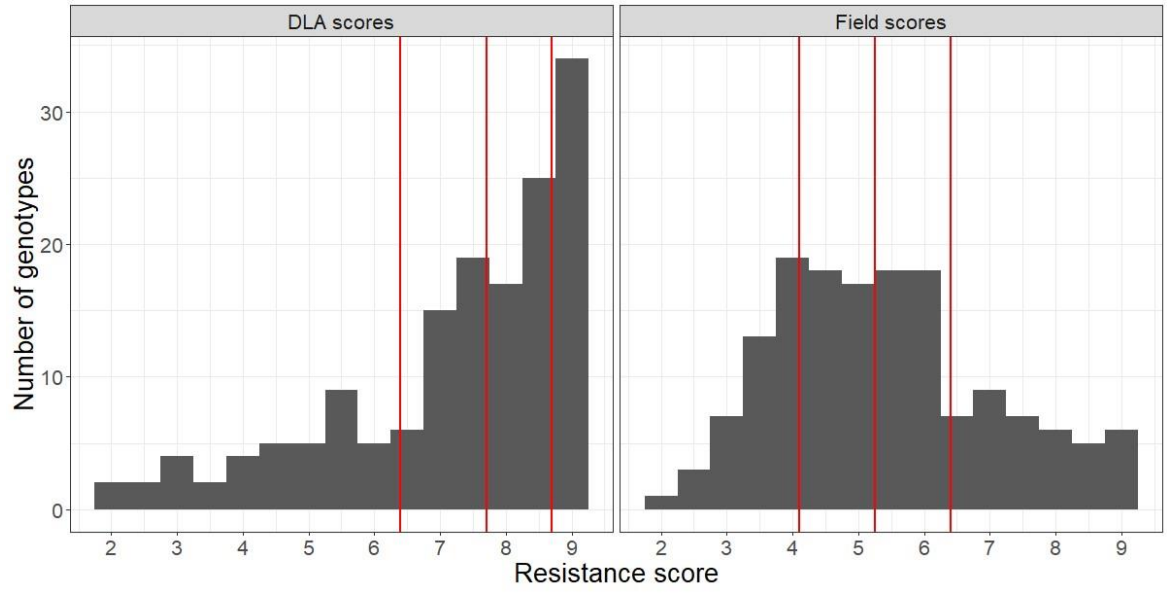

Figure 27. Histogram over the resistance scores of 154 genotypes that had been evaluated in field and used in DLA. Field scores are average of resistance scores from 2005–2021. DLA scores from using ImageJ scores from day 6 and 8. Red vertical lines indicating quartiles used for subsequent grouping. 1 = susceptible, 9 = resistant.

Table 5. Pearson (r) and Spearman (ρ) correlation between resistance scores of 152 genotypes from historical late blight field tests (2005-2021) and detached leaflet assessment. Two methods were used to convert detached leaflet assessments into resistance scores, see materials and methods. In the detached leaflet assessment genotypes were divided into three batches. Correlations were also calculated separately for every batches. Correlations not marked were significant ($p = 0.001$).

Method	All batches		Batch 1		Batch 2		Batch 3	
	Pearson	Spearman	Pearson	Spearman	Pearson	Spearman	Pearson	Spearman
Day 6 and 8	0.32	0.32	0.43	0.43	0.41 *	0.38 *	0.33 †	0.35 †
Day 6 and sporulation	0.34	0.33	0.44	0.40	0.43	0.40 *	0.35 †	0.36 †

† $p < 0.05$
* $p < 0.005$

Genotypes were grouped in categories based on quartiles (red vertical lines in Figure 27) of their resistance score (Table 6). Although the division between the categories is very simple, it shows that genotypes with a low score in DLA have a similarly low score in field trials (Table 6). However, among the genotypes with a high resistance score in the DLA there are both genotypes that were scored resistant and susceptible in field trials. The same pattern was also seen among only the overlapping genotypes.

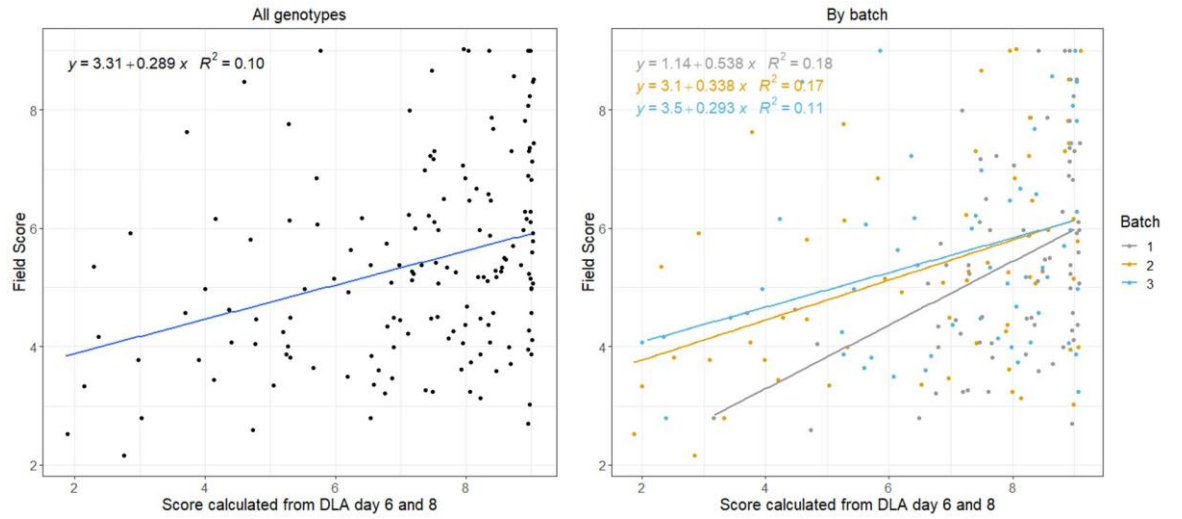

Figure 28. Comparison of resistance score from field test and detached leaflet assessment, with fitted linear regression lines. Each point represents a genotype, n = 154. Equations show the regression line formula and R^2 value. Left panel: All genotypes are evaluated together. Right panel: Genotypes are coloured and evaluated by batch.

Table 6. Comparison of genotypes that have been evaluated in field and used in DLA. Field scores are average of resistance scores from 2005-2021. DLA score from using ImageJ scores from day 6 and 8. Genotypes are evenly distributed in resistance categories based on quartiles indicated in Figure 27. Overlapping genotypes are indicated in parentheses ().

	Scores from detached leaflet assessment				
Field scores		Very susceptible	Susceptible	Resistant	Very resistant
	Very susceptible	16 (3)	9	9 (3)	5
	Susceptible	10 (1)	11 (1)	10 (1)	8
	Resistant	8	12	8	10 (1)
	Very resistant	5	7 (1)	11 (1)	15 (4)

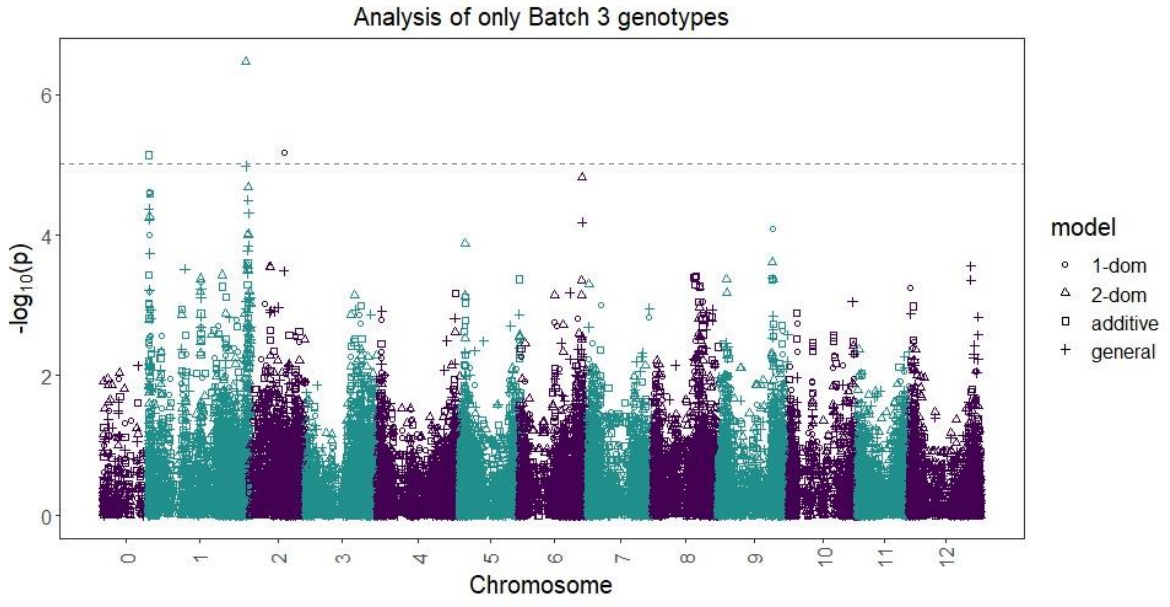

Figure 29. Manhattan plot of the analysis with only genotypes from batch 3. Stipulated threshold line for the general gene action model, for threshold of other models see Table 7.

3.3. GWAS

The GWAS revealed some significant markers. The results from the method with Day 6 % including sporulation score as phenotypic data input shown here. Significant markers are presented in Table 7. Different markers were found depending on whether all genotypes were analysed together or by batch. The markers were located on chromosome 1, 2, 3, 5, 6 and 12. Some markers only barely passed the threshold value. The Manhattan plots display the p-values of the analysis on a genomic scale along the chromosome (Figure 29 and Figure 30). Manhattan plot of batch 3 genotypes show peaks in both ends of chromosome 1, a single significant SNP in chromosome 2 and a peak in the end of chromosome 6. There were multiple significant loci in chromosome 3 between positions 42 million base-pair (Mb) and 54 Mb in the model using all batches.

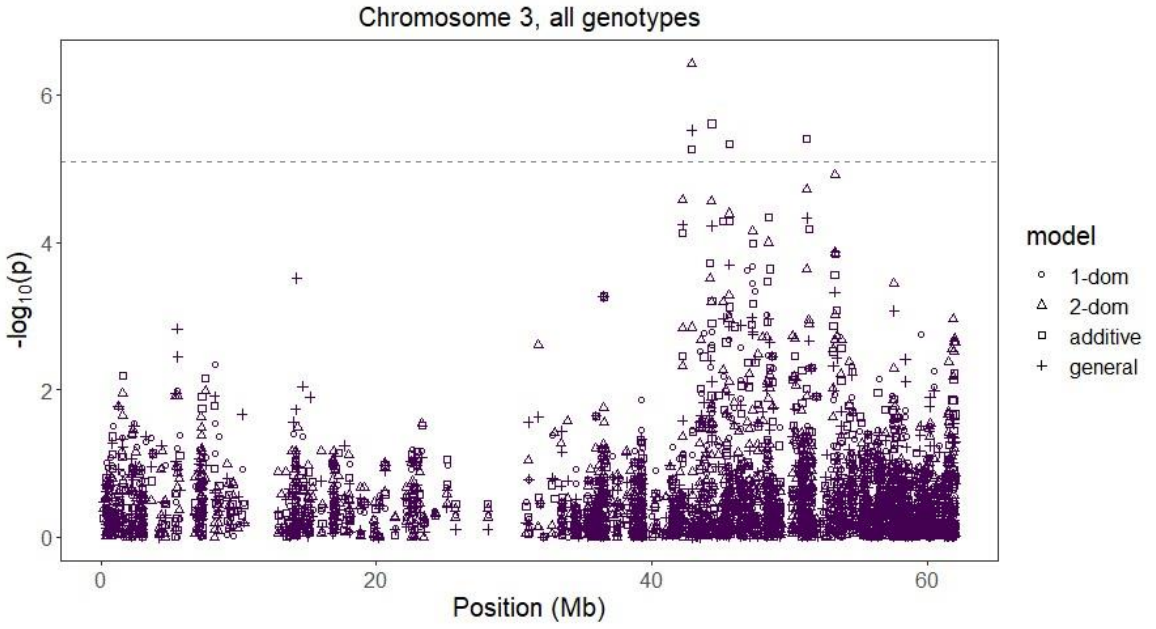

Figure 30. Manhattan plot of chromosome 3 of the GWAS where all genotypes were used. Stipulated threshold line for the general gene action model, for threshold of other models see Table 7.

Table 7. Significant markers found in GWAS. Analyses were run on all genotypes together, and separately for every batch.

Batch	Trait	Model	Threshold	Marker	Chrom	Position	Ref	Alt	Score	Effect
1	Day 6 * Mycel	2-dom-ref	4.78	solcap_snp_c1_3761	5	2957986	C	T	4.78	2.5
2	Day 6 * Mycel	2-dom-ref	4.78	solcap_snp_c1_1954	12	59371505	C	T	5.46	3.2
3	Day 6 * Mycel	additive	5.02	solcap_snp_c2_36668	1	559640	C	T	5.14	-1.2
3	Day 6 * Mycel	1-dom-alt	4.80	solcap_snp_c2_39155	2	29312995	A	C	5.17	2.0
3	Day 6 * Mycel	2-dom-alt	4.77	PotVar0036054	1	84985256	A	G	6.48	3.6
3	Day 6 * Mycel	2-dom-alt	4.77	PotVar0039866	6	53989449	A	G	4.82	2.1
All	Day 6 * Mycel	additive	5.09	PotVar0113472	3	44344721	C	T	5.61	0.6
All	Day 6 * Mycel	additive	5.09	PotVar0055831	3	51237462	G	T	5.40	-0.6
All	Day 6 * Mycel	general	5.09	PotVar0067935	3	42897873	C	T	5.52	NA
All	Day 6 * Mycel	2-dom-alt	4.89	PotVar0067935	3	42897873	C	T	6.42	2.4
All	Day 6 * Mycel	2-dom-ref	4.89	solcap_snp_c2_17631	3	53309062	A	G	4.91	-1.3

3.4. PCA plot of genetic diversity

Population structure from Structure and the principial component analysis correspond. The group relatedness from the Q matrix from Structure looks like a gradient along the first principial component (Figure 31). The 8 landraces (farmer's cultivars) genotypes, 'Rød Gullauge', 'Gullauge', 'Ingeleivs Eple', 'Shetland Black', 'Mandel 6', 'Gammel Rød Aurland', 'Gul Kvæfjærding', 'Rød Kvæfjord' and a breeding clone from a Gullauge crossing formed a small cluster in lower right corner of the score plot. To the left, 7 genotypes, 'Rosagold' culitvar and breeding clones with a similar pedigree form a small cluster. Apart from this there is no other apparent clustering of genotypes visible.

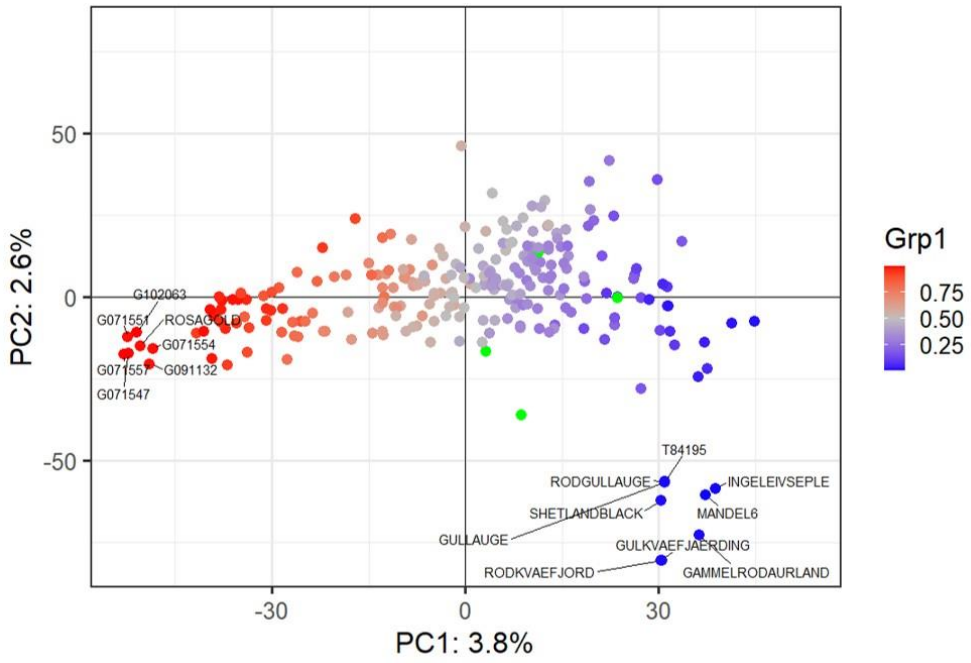

Figure 31. Score plot of the potato genotypes along PC1 and PC2 based on principal component analysis of their SNP genotypes. Potato genotypes are coloured in a gradient after their group relatedness from the Q matrix from Structure. Red = group 1, blue = group 2. Green genotypes were missing from the Q matrix.

4. Discussion

Breeding of new cultivars with improved resistance against late blight disease is an important objective in any potato breeding program. It is important that the way resistance is evaluated by the breeder reflects how the genotype will perform against late blight when the cultivar is grown commercially by farmers. Testing for disease resistant early in the breeding program is important. However, large-scale field trials are expensive, difficult to manage and control. A cost-effective alternative would be necessary to perform a preliminary test to evaluate genotypes resistance. In the current experiment we performed such a cost-effective evaluation method by growing the plants inside the greenhouse and detaching leaflets for inoculation tests with *P. infestans*; detached leaf test for foliage blight resistance (Colon et al., 2004). GWAS was also performed on the results to screen for possible significant markers. Results of the experiment were mixed as we observed some unexpected reactions in the detached leaflet assay (DLA). The results and factors that could explain some of the variation we observed are discussed below.

There was a low incidence of infected leaflets. Only 32.5 % of the leaflets that were inoculated and not discarded due to other infections showed symptoms of *P. infestans* infection. Although the incidence or infection frequency varied greatly between genotypes, as seen in Figure 18; the general incidence was low. Visker et al. (2003b) testing 6 potato cultivars got growing lesions in over 99 % of inoculated leaflets, and others doing similar experiments with multiple potato genotypes did not report any problem with incidence (Casa-Coila et al., 2019; Michalska et al., 2011; Sharma et al., 2013; Vleeshouwers et al., 1999). DLAs are also used to test and compare *P. infestans* isolates. In these experiments more variation of incidences were reported, although only a single or few different potato genotypes are inoculated (Carlisle et al., 2002; Lehtinen et al., 2009; Majeed et al., 2019). Lehtinen et al. (2009) using several Nordic isolates reported that 66–92 % and 47–62 % of leaflets from ‘Bintje’ and ‘Matilda’, respectively, sporulated depending on the isolate used. On detached leaflets of cultivar ‘Desiree’ inoculated with Pakistan isolates the incidence ranged from 59–97 % (Majeed et al., 2019). Differences in incidence between isolates are more evident on moderately resistant or resistant genotypes than in susceptible genotypes (Carlisle et al., 2002). There are also reports of isolates failing to infect any susceptible genotypes (Andrivon et al., 2011; Lehtinen et al., 2009). The incidence of an isolate are also likely to be correlated with the size of its lesions (Tooley et al., 1986). These reported results confirm that variation in incidence is to be expected between potato genotypes,

however the proportion of infected leaflets in the current experiment were lower than what have been reported before.

Dorrance and Inglis (1997) stated that inoculum failure is an inherent risk in screening evaluation, leading to escape or false designation of resistance. They argued that the reactions of the most susceptible genotypes can be used to assess this. In the current experiment we observed successful infection and large lesions on the susceptible 'G06-1033', 'Gloria', 'Hassel' and 'Colomba', but few symptoms among the other susceptible overlapping genotypes 'Bintje', 'Mandel 6' and 'Eersteling'. The concentration of sporangia/ml in the inoculum can also affect the incidence. Hodgson (1961) saw higher incidence with higher concentration. However, it will only increase until a certain point before decreasing possibly due to lack of oxygen for the sporangia (Sobkowiak et al., 2004). Two concentrations were used in this experiment, 15 000 and 34 000 sporangia/ml, both with 50 µl inoculum droplets. Highest incidence on the overlapping genotypes were observed in batch 2 with 34 000 sporangia/ml, but between batch 1 with 15 000 sporangia/ml and batch 3 with 34 000 sporangia/ml the infections on overlapping genotypes were about equal. Concentrations ranging from 3000 sporangia/ml using 50 µl droplets to 500 000 sporangia/ml using 20 µl droplets can be found in the literature (Casa-Coila et al., 2019; Sharma et al., 2013). Stewart (1990) noted that optimal inoculum concentration likely depends on the isolate of *P. infestans*. It is possible that we could have achieved higher infection frequency and less ambiguously results by using higher concentrations.

Another factor possibly affecting the inoculum quality is the age. In our experiment *P. infestans* were transferred to the Rye B Agar plates 8 to 10 weeks before the inoculation experiment. There are reports of using agar plates for maintenance of isolates and transferring to new plates every 3 or 6 months (Karki & Halterman, 2021; Vega-Sanchez et al., 2000). However, when collecting sporangium for inoculation the plates used are usually between 7 to 14 days old (Karki et al., 2021; Kuhl et al., 2007; Vleeshouwers et al., 1999). Old sporangia are reported to have a lower percentage of germination and develop a large vacuole (King et al., 1968; Leesutthiphonchai & Judelson, 2019). Large vacuoles in some sporangia and a low percentage germination were observed in this study. The infectivity of these older sporangia is uncertain, but likely lower than younger sporangia. Sporangia are earlier shown to remain infective for 8 weeks in soil (Lacey, 1965). A way of determining the frequency of infectious sporangia are proposed by Lehtinen et al. (2009). Their method was adapted from the most probable number

technique from bacteriology. They produced a suspension of 10^4 sporangia/ml and serially diluted an aliquot of the suspension four times by a factor of four, resulting in suspensions of 2500, 625, 156 and 39 sporangia/ml. Leaf discs cut out with a cork borer were then inoculated with a 10 µl droplet of the suspension, 10 disc per suspension. Using the number of sporulating discs they calculated the frequency of infectious sporangia. However, the results from the method should be interpret with caution, as the test method seem to underestimate the actual frequency (Lehtinen et al., 2009). This method could have revealed how the age could affect the virulence of the inoculum.

There was a lack of information about the isolate or possible isolates of *P. infestans* used as inoculum. It is uncertain how the recurring transfers to new potato tuber slices every week for a year affected the original isolates. Perhaps some isolates were lost as other isolates outgrew them on the slices. The 12-plex SSR genotyping did not match our isolate with any known genotype, but there were high similarities with the EU_41_A2 genotype (Appendix 3). The differing alleles on locus Pi04 have been seen earlier in Nordic populations of *P. infestans* and allele 215 in locus Pi4B have been reported in the Netherlands, but both are seemingly quite rare (Li et al., 2013; Sjöholm et al., 2013). The *P. infestans* population in the Nordic region is highly diverse with many unique multi-locus genotypes, so a genotype without any match was not unexpected (Brurberg et al., 2011; Puidet et al., 2022). The inoculum could also be characterized through determining its mating type and virulence against race-specific resistance genes. Virulence can be tested after the protocol of Andrivon et al. (2011) on Black's differential set of 11 potato cultivars with different R genes (Malcolmson & Black, 1966; Zhu et al., 2015). Mating type can be determined by pairing the unknown isolate with a known isolate of A1 or A2 on a Petri plate with nutrient agar and look for oospore formation after 2-4 weeks (Galleby & Galindo, 1958; Lehtinen et al., 2008). It is beneficial with an isolate of a complex race that can overcome race-specific resistance. The observed differences in resistance of leaflets between potato genotypes can then be attributed to field resistance, and not race-specific resistance (Stewart, 1990). Another improvement of our experiment would have been to use more than one isolate as inoculum. Isolates vary in aggressiveness and the ranking between potato genotypes could depend on the isolate (Casa-Coila et al., 2019). It is also important to note that some isolates are reported to perform inconsistent in inoculation trials. Andrivon et al. (2011) speculated that this might be due to the genetic structure of these isolates.

Propagation of inoculum on Rye Agar plates is an efficient technique also used by others (Karki et al., 2021; Kuhl et al., 2007; Stephan et al., 2005). An alternative is to harvest sporangia from infected leaflet or tubers inoculated in advance (Colon et al., 1995a; Michalska et al., 2011; Warren et al., 1971). Authors often stated that they harvest from potato tissue to restore virulence of the pathogen if it has been maintained on agar. Some reports that using several passages on host tissue is more beneficial than single or double passes (Sobkowiak et al., 2004). A group of scientists at Cornell University strongly advise against using artificial media to propagate sporangia of *P. infestans* for inoculation trials, stating that such sporangia is significantly different of sporangia from host tissue. Sporangia from artificial media are reported to be less aggressive, produce less disease, lower rates of indirect sporulation and have differences in gene expression (Fry et al., 2019). These claims are refuted by scientists from University of California, who could not observe said differences, but emphasized the importance of following "best practices" (Leesutthiphonchai & Judelson, 2019). They recommend using a modified Petri's solution instead of deionized water for harvesting and diluting sporangia from plates (Leesutthiphonchai & Judelson, 2019; Petri, 1917). Petri's solution reportedly reduces batch-to-batch variation in germination of sporangia by replenishing leached ions from sporangia and buffering pH. It is uncertain whether the 1 % potato tuber extract used in the current experiment provided the same benefits. The discussion regarding sporangia from artificial plates highlight the sensitivity and cautiousness that successful inoculum preparation demands. Inoculum preparation can be a large source of batch-by-batch variation as others have experienced (Lehtinen et al., 2009; Ordoñez et al., 1998).

Ideally all variation observed between potato genotypes should be a result of differences in resistance. However, several factors could possibly affect the observed resistance in detached leaflet assay and their contribution to variation in the test should be limited to a minimum. The physiological age of the tubers can affect the observed resistance, older tubers have been more susceptible than younger (Stewart et al., 1983). In our experiment these differences were minimized by producing the majority of the tubers on the same field and storing them all together. Another option is using in vitro plantlets instead of seed tubers (Rubio-Covarrubias et al., 2005; Visker et al., 2003b; Vleeshouwers et al., 1999). Visker et al. (2003b) cited Lommen (1999) on that in vitro material develop sooner than material from tubers. This should be considered if comparing plant age at testing. On the other hand, some report no difference in performance in DLA between plants from tubers and in vitro (Andrivon et al., 2011). Another benefit is that in vitro material is likely to be clean of viruses, which are conflicting reported to

make genotypes both more susceptible and resistant in detached leaflet assays (Lin et al., 2014; Pietkiewicz, 1974). In the current experiment the genotypes were divided into batches alphabetically. The maturity of genotypes are reported to be closely linked to resistance against late blight, with early maturing genotypes being the most susceptible (Bradshaw et al., 2004). Therefore, it is beneficial to group genotypes by maturity if they cannot all be assessed at the same time (Bradshaw, 2021).

The growth conditions in the greenhouse can affect resistance. In our experiment we only used a single plant per genotype. Ideally the environment should be equal for every plant. However, it is difficult to ensure this equality. For example, plants on the edges of the table in the greenhouse will have less competition than those in the middle. Sufficient plant spacing were a problem in our experiment due to space limitations, and all plants became straggly with long weak stems. Straggly plants are reported to be less resistant than sturdy well-spaced plants (Stewart et al., 1983). Water stress can also affect observed resistance (Darsow et al., 1988; Warren et al., 1971). Without any replications in form of extra plants per genotype it is impossible to assess whether this affected the results of the current experiment. Plants should be of similar age when leaves are tested. Rate of lesion growth are reported to decrease as plants mature, before they become very senescent and susceptibility increase (Carnegie & Colhoun, 1982). In the current experiment all plants were 8 weeks old when leaflets for inoculation were harvested, but there were differences in plant stage among genotypes. Other environmental factor that are known to affect resistance, but should affect all plants equally are day length, time of day of sampling, duration of sunshine the 1–3 days before sampling, temperature and season of growth (Darsow et al., 1988; Rubio-Covarrubias et al., 2005; Stewart, 1990). These factors could however contribute to differences between batches and repeated experiments.

The sampling of leaves and leaflets are another possible source of variation in resistance response among the genotypes. Leaves from the upper two-thirds of the plant were primarily chosen. As the picking of leaves were done in a separate operation apart from leaflet selection it is possible that the actual position could vary between genotypes. Leaf position affects the growth of lesions. Resistance increase linearly from leaves at the base of the plant to leaves at the apex (Carnegie & Colhoun, 1980). However, the differences in resistance seem to be negligible in leaf position 10 and upwards from the base (Visker et al., 2003b). Leaflets on the same leaf can exhibit different reactions (Carnegie & Colhoun, 1982). In our experiment we prioritized selecting lateral leaflets, but also apical leaflets are used by some (Njoroge et al.,

2019; Rubio-Covarrubias et al., 2005). A more systematic approach to the collection of leaflets could have given enough traceability to assess whether this affected our results.

Inoculated leaflets can be assessed in many ways. Scoring after percentage infected leaflet area and degree of sporulation was a quick method that allowed all genotypes in a batch to be scored in a day. The good correlation between visual scoring by eye and through manual image analysis in ImageJ suggest that both are viable methods. The main benefit of scoring by eye was that it did not require further evaluation and processing of images at a later stage. The biggest drawback was that it lacked the time perspective that scoring sequentially images from multiple days offered. On the other hand, taking images were quicker than scoring by eye, but subsequent operations made it more time consuming. Assessments in ImageJ were more accurate as only the distinction between infected and uninfected tissue were subject to biased evaluation. When scoring by eye, the scorer also had to determine the proportion of infected area, which in ImageJ were calculated automatically. Even though manually drawing lesion boundaries in ImageJ is a big improvement from Tooley et al. (1986)'s method of sketching the lesion with a pencil and using a planimeter to calculate the area, it is possible to speed up these operations further. A red calibration square could be used for scale calibration instead of manually drawing a line along the ruler in ImageJ (Easlon & Bloom, 2014), algorithms can recognize leaflets in a Petri dish or leaflets of a compound leaf (Failmezger et al., 2018) and boundaries between infected and healthy tissue can be recognized automatically (Mandal et al., 2021; Mukherjee, 2020). Unfortunately, no single program with all these functions exists for the moment. When assessing the degree of sporulation, it was difficult to distinguish between moderate and intense sporulation. How efficient the pathogen can multiply itself is an important trait that should be scored precisely. This could have been assessed more precisely by washing of the spores of infected leaflets into a known volume of water, determine the concentration and divide it by the lesion area (Kuznetsova et al., 2014; Lin et al., 2014). An alternative to scoring the area affected by the pathogen would be scoring the linear lesion growth rate in mm/day which is shown to give good correlation with field data (Colon et al., 1995b). This value should not differ much from the percentage score of even sized leaflets but would be more accurate when leaflet sizes are variable.

Our data show benefits of assessing the leaflets multiple times. Infections on some leaflets developed slowly with only minimal symptoms 6 days after inoculation. Assessing multiple times requires more labour and is a question of cost-benefit. It is not certain that scoring multiple

times are necessary for distinguishing susceptible and resistant genotypes. The calculated 1–9 resistance score between using only day 6 and using day 6 and 8 were very similar. However, if the leaflets will only be assessed once it would be beneficial to do it at day 6 in our experiment, although this is likely to depend on the tested genotypes and the inoculum. At day 8 many leaflets were fully covered with mycelium making it more difficult to distinguish between susceptible genotypes.

In this experiment, the correlation between resistance scores from breeding field trials and the detached leaf test was limited. Correlation ranged from 0.32 to 0.34 depending on method when looking at all genotypes together. Evaluating genotypes batchwise yielded a higher correlation for batch 1 and 2 (r = 0.41–0.44), but not for batch 1 (r = 0.33–0.35). High dispersion from the regression line in the scatter plot makes any direct conversion of scores between the two methods difficult. High dispersion when comparing individual genotype results from DLA and field trials were also observed by Zweep (2014). As discussed earlier, there was a low frequency of infections on leaflets likely due to problems with the inoculum. Many genotypes therefore got a high resistance score from the DLA. Artificial grouping into four resistance categories show that the detached leaflet method identified many of the most susceptible and resistant genotypes, even with the inoculum problems. Correlation between field trials and laboratory methods of assessing late blight has been investigated earlier. Some report finding no correlation (Haynes et al., 2007), but the many observed some correlation (Dorrance & Inglis, 1997; Malcolmson, 1969; Michalska et al., 2011; Rogozina et al., 2010; Sharma et al., 2013; Singh & Birhman, 1994; Umaerus & Ihnell, 1976; Visker et al., 2003b; Vleeshouwers et al., 1999; Zweep, 2014). However, some report finding higher correlation with field test through inoculation of stems (Michalska et al., 2011) or whole plants (Dorrance & Inglis, 1997; Sharma et al., 2013) than using detached leaves and leaflets. Except for Haynes et al. (2007) (n = 69), Rogozina et al. (2010) (n = 78) and Zweep (2014) (n = 104), all the cited experiments used less than 25 genotypes. Two recently published papers screened resistance in 189 genotypes of 20 wild species and cultivated potato, and 894 plants from true potato seeds of 72 wild potato species with detached leaf assay (Duan et al., 2021; Karki et al., 2021). This shows that evaluation of many genotypes is feasible.

The results of this study indicate that DLA can be useful in a breeding program. The breeder can identify genotypes that are very susceptible to late blight and reject these before performing field trials. This opinion of laboratory assays is also shared by others (Dorrance & Inglis, 1997;

Michalska et al., 2011; Sharma et al., 2013; Stewart et al., 1983). In the current study it was difficult to distinguish resistant and moderately susceptible genotypes. As discussed, there are several factors that could contribute to variance in resistance, apart from just the innate resistance of a genotype. It is possible that controlling these factors would improve the accuracy and efficacy of the assay, allowing the breeder to reject even more genotypes. However, based on this study and what is reported by others, field tests will still be the most accurate and important method to assess foliage late blight resistance (Michalska et al., 2011; Sharma et al., 2013).

One of the significant markers found in the GWAS did match a reported resistance gene against foliage late blight. The thresholds for significance were not adjusted for multiple testing in the current experiment and an artefact of this is an increased risk of false positives. QTL markers for late blight resistance have earlier been found on chromosome 3. The significant SNP markers from this study PotVar0113472 and PotVar0067935, close to positions 4.43 Mb and 4.29 Mb, are located near a QTL tagged between RFLP markers GP25 and CP6 (Gebhardt & Valkonen, 2001; Santa et al., 2018). Another GWAS also found significant SNP markers against late blight in this region, close to positions 4.55 Mb (Lindqvist-Kreuze et al., 2021). No known QTLs could be found in proximity of the other significant markers, although there are other QTLs on the same chromosomes (Ewing et al., 2000; Leonards-Schippers et al., 1994; Santa et al., 2018; Visker et al., 2003a). An earlier GWAS of the field data from Graminor has revealed a significant marker at chromosome 11 (unpublished), but no significant marker in this region were found from the DLA data.

Very limited population structure was found between the potato genotypes used. The analysis in Structure suggested two subpopulations, but many genotypes were equally associated with both subpopulations, as seen in the score plot. Our observations are in line with a recent study of population structure in potato accessions from the Nordic genebank (n = 75) and breeding material from the Swedish potato breeding program (n = 49) (Selga et al., 2022). Landraces genotypes clustered together in their study, similar to what was observed here. Weak population structure has also been found other studies of cultivated potato (Sharma et al., 2018).

Late blight resistance in 255 potato genotypes were assessed through detached leaflet assay (DLA). There were problems with low frequencies of infection among leaflets of several genotypes, possibly due to subpar inoculum. This highlights the importance of minimizing the impact of factors that contribute to variation in detached leaflet assays, apart from genotype

resistance. Despite this, the most susceptible genotypes were identified in the DLA. Genome-wide association study found several significant SNP markers, and one of the markers was located close to a previously mapped QTL against late blight.

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6. Appendix

6.1. Appendix 1: Genotypes with multiple tubers

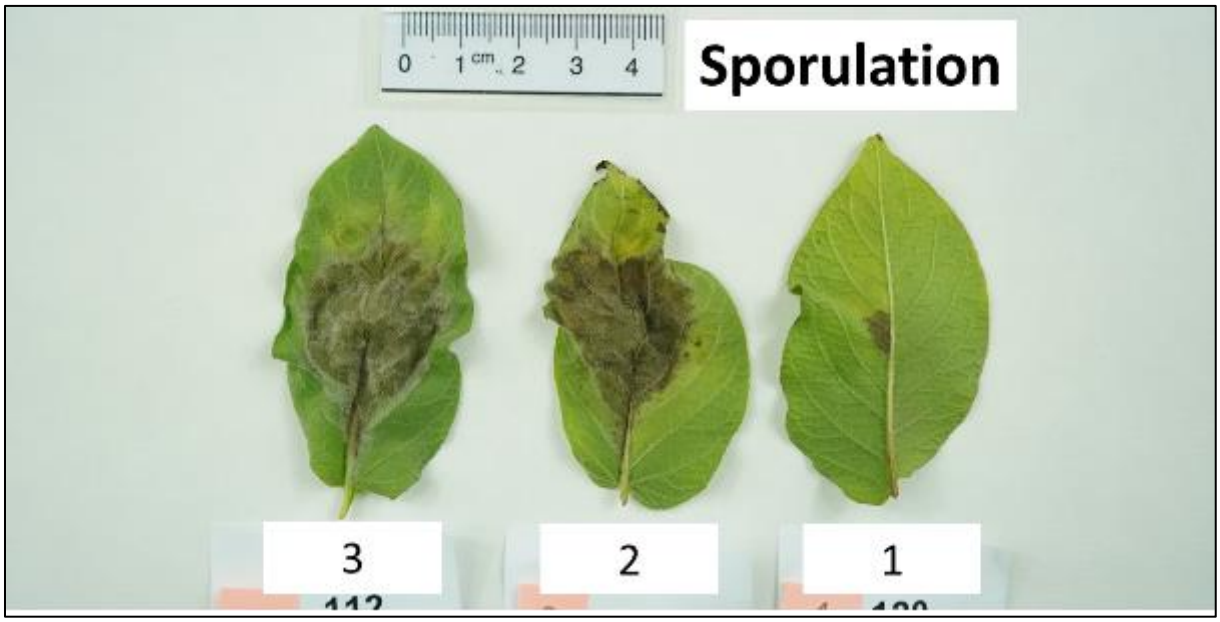
Genotypes planted with multiple tubers in the pots to ensure sprouting and growth.

Genotype	Batch and No.	No. of tubers
Sparris	3-245	3
Ringerikspotet	3-227	2
Prestkvern	3-224	4
P01-5-72	3-207	3
NCT-92-22-14	3-191	4
N-84-6-95-1-2	3-182	2
N-42-15-138	3-184	2
Marius	3-176	4
Carolus, ny	3-026	2
G11-1301	2-139	2
G11-1495	2-140	2
G09-9081	2-116	2
Carolus, ny	2-026	2
Carolus, ny	1-026	2
Canberra	1-024	3
Anouk	1-006	2
ARD01-2268	1-007	2
Arielle	1-009	2

6.2. Appendix 2: Scales for sporing infections

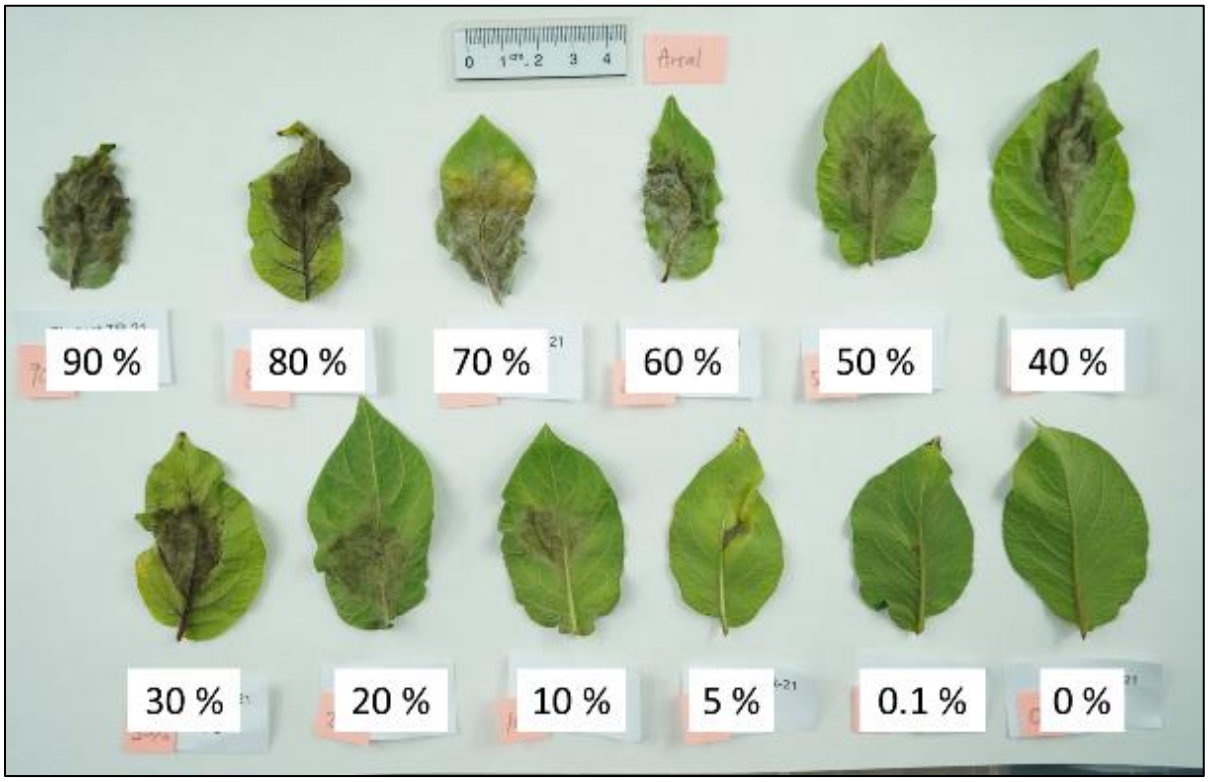
6.2.1. Sporulation scale

Scale for scoring sporulation of *P. infestans* on leaflets. 1 = no sporulation; 2 = slight to moderate sporulation; 3 = intense sporulation.



6.2.2. Infected area

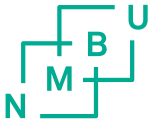
Scale for scoring percentage infected leaflet area.



6.3. Appendix 3: *P. infestans* genotype

Results of 12 plex-genotyping *P. infestans* isolate(s) used as inoculum in the experiment. EU_41_A2 genotype as reference. Differing alleles coloured yellow.

Genotype	**Locus**	**Pi02**		**Pi4B**		**G11**		**Pi04**		**Pi63**		**Pi70**		**D13**		**SSR11**		**SSR2**		**SSR4**			**SSR6**		**SSR8**	
	Allele	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	3	1	2	1	2
Graminor		266	268	213	215	156	206	166	174	279	279	192	192	136	136	341	341	173	175	284	288	294	242	244	260	266
EU_41_A2		266	268	213	213	156	206	168	168	279	279	192	192	136	136	341	341	173	175	284	288	294	242	244	260	266



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