

# Characterisation of *Podosphaera pannosa*, rose powdery mildew

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## Introduction

Roses (genus *Rosa*) are the most sold flowers in the world. FloraHolland, the world's largest flower auction, sold 3.7 billion stems with a total value of €761 million in 2011. Besides cut roses, 39 million pot roses were sold for €45 million and 2 million garden roses were sold for €6 million (FloraHolland 2011). The major threat in the rose industry is powdery mildew, it is the most occurring pathogen on rose in Europe and the United States. There is a wide variety in powdery mildew species but this study will focus on rose powdery mildew (*Podosphaera pannosa*). *P. pannosa* used to be known as *Sphaerotheca pannosa*, for clarity purposes only *P. pannosa* will be used in this report. The first record of powdery mildew dates back to 300 B.C. making it one of the oldest recorded plant diseases (Watkins 1990).

Chemical fungicides are widely used to prevent and cure powdery mildew infections but with the growing tendency to reduce the use of chemicals in agriculture, these are becoming less of an option. More and more focus is put on resistance breeding in rose against powdery mildew.

## Powdery mildew

*Podosphaera pannosa* is an obligate biotroph fungus that grows on Rosaceae. There are many more fungi that are called powdery mildew and they all have different host ranges. The fungus is a member of the Ascomycota and belongs to the order of Erysiphales (Hückelhoven and Panstruga 2011), it occurs in most climates with moderate temperature. The spores are spread by wind and require high relative humidity to germinate. Liquid water however kills the spores, the developed fungus is also sensitive to heat and direct sunlight (Gubler and Koike 2009). The main symptom of a *P. pannosa* infection is a white cotton like growth of mycelium on the leaves and flower buds. Powdery mildew is not lethal to the plant but it gives a reduction in growth and aesthetic value and thereby diminish commercial value (Kaufmann et al. 2012).

Powdery mildew spores are carried by wind, when they land on a leaf, the spores germinate. After germination, a penetration peg is formed which makes it possible for the fungus to penetrate the cell wall. When the fungus has entered the cell, a haustorium is formed to take up the nutrients from the cell and provide nutrition for the fungus. The fungus now grows out over the leaf, covering it completely in the well known white cotton like mycelium. After about three to five days new conidiophores are formed which release new spores to repeat the cycle. This process is known as the vegetative life cycle (Kaufmann et al. 2012).

The fungus overwinters by forming cleistothecia that grow in infected flower buds. The process of forming those cleistothecia happens at the point where normally the conidiophores are formed. The cleistothecia are more resistant to extreme conditions and when they land between fallen leaves they can survive the winter (Gubler and Koike 2009).

Resistance against powdery mildew is found in *Arabidopsis*, barley and pea (Hückelhoven and Panstruga 2011). Resistance against powdery mildew requires the deletion or mutation of the powdery mildew locus O (MLO). This locus has been found in rose but there are two major difficulties with it. First of all, the locus is very close to the double flower gene, with all cut and pot roses having double flowers, this poses a problem. Because of the linkage between the MLO gene and the gene that is responsible for the double flowers in rose it is statistically highly unlikely that a cross between those genes will occur in a meiosis event. This makes it almost impossible to get a double flower rose with a disrupted MLO gene as the result of a cross between a commercial cultivar and a plant with a disrupted MLO gene. The MLO gene needs to undergo a loss of function mutation to provide resistance against powdery mildew. This was proven by experiments done in *Arabidopsis* (Hückelhoven and Panstruga 2011). There are however no MLO genes found in rose that have such a loss of function mutation which makes it impossible to develop a powdery mildew resistant rose using the MLO gene (Kaufmann et al. 2012).

Besides the MLO based resistance there is also resistance based on the *Mlg* gene. This gene was first discovered in barley and inhibits the growth of *Blumeria graminis* f. sp. *Hordei* by triggering the release of hydrogen peroxide in the infected cell causing it to die (Hückelhoven et al. 1999). This hypersensitive response is very effective against a biotroph as powdery mildew. Recent research has shown that the *Mlg* gene can also be found in roses and provide resistance against *P. pannosa* (Dewitte et al. 2007).

A similar resistance mechanism is imposed by the *Mla12* gene, also found in barley. This gene also causes a hypersensitive response using hydrogen peroxide but comes into action 24 to 40 hours post infection while *Mlg* expresses 14 to 24 hours post infection (Huckelhoven et al. 1999).

## Internal transcribed spacers

The internal transcribed spacer is a region in the ribosomal DNA (rDNA). Takamatsu et al. (1998) concluded that the ITS might be involved in creating the secondary structure of the 18S and 26S DNA located next to the ITS. The function of the ITS is however very little which makes it subject to fast evolutionary divergence (Schlötterer et al. 1994). The ITS is therefore a highly variable site in the fungal genome. Because of this variability, the ITS is widely used in evolutionary studies in fungi (Lücking et al. 2013). With the ITS located in the rDNA, there is a high copy number in each cell, due to the many copies of the ribosomes, this makes it easier to recover the sequence from small sample sizes with little isolated DNA. A lot of research has been done on the ITS of many different fungi, therefore a large database with sequences is available. The NCBI database contained over 900.000 entries at February 1, 2014. The large database makes it possible to identify a large number fungal species by their ITS. The variability in ITS can also be used to identify different isolates within *P. pannosa* (Leus et al. 2006). The ITS itself does not have any influence on the phytopathological properties of powdery mildew but it can be used as an identifier for different groups of powdery mildew. Each of the different groups, identified by the ITS, can have different properties concerning host range and virulence.

## Disease test

To test whether or not a plant is resistant to *P. pannosa*, a disease test has to be performed. These tests are usually done on a population of full grown plants (Yan et al. 2006) or a representation of that population. It is however very costly to do such an experiment on a population of adult plants due to costs of greenhouse space. A more effective way could be to do a disease test on detached leafs. These experiments can be done in growth chambers and require only a fraction of the space. Positive results have been achieved with this system in late blight in potato (Vleeshouwers et al. 1999). Efforts will be made to introduce this system in rose to investigate powdery mildew resistance.

## Current problems in tissue culture

Before this project was started there was already a project with the goal of culturing powdery mildew on *in vitro* roses. The results of this research were however minimal, there was hardly any visible powdery mildew growing on the leaves of the plantlets. As mentioned before, powdery mildew spores get killed when they come into contact with liquid water. The conditions in the plastic container and in the growth chamber caused a lot of liquid water on the inside of the containers in the form of condensation.

## Aim of the research

With this research project, we aim at developing a reliable DNA isolation protocol supplying a sufficient amount of powdery mildew DNA enabling amplification of the ITS regions by PCR. The amplicons obtained from the different isolates will be then sequenced. By comparing the sequences of the different isolates, hopefully we will be able to distinguish between isolates based on their ITS sequence.

Besides the identification of different powdery mildew isolates we also aim at developing a simple and cheap way of maintaining the isolates. The isolates need to be maintained on living roses and when the isolates are separate we need to make any cross contamination between them impossible. The yield of the culture method should be high enough to not only maintain the culture on fresh plants but there should also be enough material to use in resistance tests and for molecular research.

The two goals described above should pave the way to research focusing on natural resistance against powdery mildew in roses. With this larger goal in mind we shall also make effort in developing a cheap and simple disease test for powdery mildew in roses. Screening on a large scale shall not be done due to time limits

## Materials and methods

### *P. pannosa* strain origins

Powdery mildew from three countries were used in the experiments. Two isolates from Germany were provided by professor Thomas Debener originating from outdoor roses. They were maintained separately in vitro, however after arrival in Wageningen could not be kept separate but were maintained as a mixture, further referred to as the 'Hannover' isolate. Three other isolates originated from Belgium and were provided by Leen Leus. Those isolates were obtained from in vitro cultures (two) and from an outdoor garden rose. Also these three strains could not be maintained separately in Wageningen and were grown in a Weiss cabinet as a mixture, further referred to as the 'Melle' isolate. From the Netherlands three possible strains were isolated. The first strain was found on a rose population in the WageningenUR greenhouse (WUR isolate), the second strain came from the greenhouse of the Lex+ rose breeding company (De Lier isolate) and the third strain originated from the greenhouse of the Terra Nigra rose breeding company (De Kwakel isolate).

### *P. pannosa* maintenance

The Hannover and Melle powdery mildew isolates were maintained in two separate Weiss cabinets (WeissTechnik). The Weiss cabinets were used to maintain a stable climate for both the rose as well as the powdery mildew. The temperature in the Weiss cabinets was set to 22°C and the relative humidity (rh) was set to 75%. The powdery mildew was maintained on roses from the K5 population. The K5 population consist of 181 plants as a result of a cross between two tetraploid roses and it was used in the past to test for resistance to powdery mildew and to localize molecular markers on the genetic map (Yan et al. 2006). The powdery mildew used in these experiments were all maintained on the K188 genotype which previously appeared to be susceptible to powdery mildew and therefore proved to be an excellent plant to maintain the powdery mildew on.

### DNA isolation

Two different DNA isolation methods were used and evaluated in this project. Firstly, Chelex 100 resin (Bio-Rad) according to a published protocol (Saenz and Taylor 1999). Clippings of a rose leaf (approximately 100mg) that was infected with powdery mildew were mixed with a 5% w/v Chelex (Walsh, Metzger, and Higuchi 2013) suspension and autoclaved (121°C, 2 atmospheres). The solution was centrifuged and the DNA remained in the supernatant. The Chelex resin is capable of binding multivalent metal ions. These metal ions are released by the tissue when the tissue is autoclaved. The metal ions play a catalysing role in the breakdown of DNA in high temperatures, because the Chelex binds these ions they are no longer available for catalysing the DNA breakdown and thus the DNA is still usable for a PCR (Walsh et. al 1991).

Secondly, the Wizard Magnetic DNA purification system for food (Promega) was tested and evaluated. The system which works by binding DNA to small magnetic beads was used according to the manufacturer's instructions. DNA has a natural affection for silica, the MagneSil particles used in the Wizard kit are made of magnetite ( $Fe_3O_4$ ) and covered in a thin layer of silica (Bishop and Condor 2011).

About 100 mg of material was taken per sample. The protocol uses a beadbeater with a 3mm stainless steel bead to disrupt the material. This method works very well for the fungal part of the material but not so well for the plant cells. Because of this the ratio fungal DNA/plant DNA was favored in the direction of the fungal DNA. Still we ended up with a mixture of both plant DNA, powdery mildew DNA and DNA from other organisms growing on the leafs but with the sensitivity of the PCR this did not pose a problem.

The first batch of powdery mildew samples came from four different sources. There were samples taken from two Weiss cabinets, one containing the Melle isolate and one Weiss cabinet contained the Hannover isolate, from each Weiss cabinet three separate samples were taken. In the greenhouse, there was a collection of rose progeny plants made from assorted crosses by Carole Koning. Here, plants also suffered from a natural powdery mildew infection and three separate samples were taken from those plants, the Wageningen isolate. As a control samples were taken from healthy, non-infected plants (Wageningen Control). From the Wageningen Control population three samples were taken from three genotypes, one sample per genotype. The two parental genotypes (P540 and P576) were chosen as healthy control and the genotype on which the mildew was maintained in the Weiss cabinets (K188) was chosen as a healthy control. DNA was isolated no more than one hour after picking. The infected leaves

were chosen for their high infection density and it was also made sure that the mycelium was white indicating that the mycelium was young.

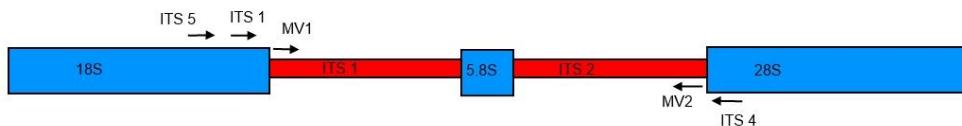
Two breeding companies, Lex+ and Terra Nigra, also kindly sent powdery mildew samples from their greenhouses. From those samples (De Lier and De Kwakel isolates) DNA was isolated as described earlier.

## PCR

To test the primers, a semi nested PCR using the DreamTaq polymerase (Thermoscientific) was used. The reaction mixture of the first part of the semi-nested PCR consisted of 2  $\mu$ l isolated DNA, 2  $\mu$ l 10x taq buffer, 0.04  $\mu$ l DreamTaq polymerase, 0.4  $\mu$ l forward primer (10  $\mu$ M), 0.4  $\mu$ l reverse primer (10  $\mu$ M), 0.4  $\mu$ l dNTP's (10  $\mu$ M) and 14.76  $\mu$ l MiliQ water to come to a total reaction volume of 20  $\mu$ l. The following thermo-cycling profile consisted of 10 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 65°C and 2 min 72°C followed by a final extension step at 72°C for 10 minutes (Leus et al. 2006).

The PCR primers were also used by Leus et al. (2006) but originate from White et al. (1990). In the first part of the nested PCR, we used the ITS1f primer (5'-CTTGGTCATTAGAGGAAGTAA-3') as forward primer and the ITS4r primer (5'-TCCTCCGCTTATTGATATGC-3') as reverse primer. In the second part of the nested PCR we used the ITS5f primer (5'-GGAAGTAAAGTCGTAACAAGG-3') as forward primer and the ITS4r as reverse primer. The thermo-cycling profile was modified to an annealing temperature of 60°C.

A second primer set (MV) was developed by myself based on available sequences of *P. pannosa* and rose. I designed this primer set to be more specific than the primers in the article of Leus et al. (2006). The MV primers annealed to a site conserved in the powdery mildew ITS. MV ITS1f (5'-TGAAGTGAATTGTTGCTTGGC-3') was used as forward primer and MV ITS2r primer (5'-TATCCCTACCTGATCCGAGG-3') was used as reverse primer. The PCR program consisted of a standard program used for the taq polymerase (Thermo Scientific) and consisted of 5 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 56°C and 1 min at 72°C, followed by a final extension step at 72°C for 7 minutes.



**Figure 1 schematic overview of the internal transcribed spacer with the MV primer locations and the Leus (ITS) primer locations**

Two other primer sets (Podo 1 and Podo 2) were developed by Els Verstappen and Theo van der Lee also based on available sequences of rose and *P. pannosa*. The available sequences of rose and powdery mildew were taken from the BLAST database and aligned. We looked for a region in the ITS that was identical for all powdery mildew but unique for rose. That region then was put in the BLAST database to check if the chosen sequences was also unique against other fungi. Primers were picked and using Primer-BLAST and Ape it was checked for annealing temperature and self-looping to ensure the quality of the primers.

The Podo1f (3'-TTGTCCCTGCGCGGCT-5'), Podo1r (3'-ATACGTAGACTACGCGG-3'), Podo2f (3'-GCTGACCCCTCCACCC-5') and the Podo2r (3'-GACCGCCAGCTGTTT-5') primers were used with the same PCR program as the MV primers, the annealing temperature was the same as the MV primers. All the primer pairs are located near each other and the expected length of the product is around 430 base pairs.

For the PCR product that was going to be sequenced we used the more accurate proofreading Phusion DNA polymerase (Qiagen) for the first part of the nested PCR. The reaction mixture of the first part of the PCR consisted of 20 ng isolated DNA, a nanodrop spectrophotometer was used to determine the concentration, 4  $\mu$ l 5x Phusion HF buffer, 0.5  $\mu$ l Phusion polymerase, 1.0  $\mu$ l forward primer (10  $\mu$ M), 1.0  $\mu$ l reverse primer (10  $\mu$ M), 0.8  $\mu$ l dNTP's (10  $\mu$ M), depending on the DNA concentration MiliQ water was added to a total reaction volume of 20  $\mu$ l. The PCR program consisted of 30 sec at 98°C, 35 cycles of 10

sec at 98°C, 1 min at 65°C and 20 sec at 72°C, followed by a final extension step at 72°C for 10 minutes.

The second part of the nested PCR was done using the Advantage DNA polymerase (ClonTech), this enzyme enables to ligate the product in a vector for cloning by adding sticky ends to both ends of the PCR product. These ends are specifically designed to be compatible with the T Easy vector system (Clontech Advantage user manual). The PCR reaction was done in a total volume of 50  $\mu$ l. the reaction mixture consisted of 5  $\mu$ l 10x Advantage buffer, 2  $\mu$ l dNTP's (10 mM), 2  $\mu$ l forward primer (10  $\mu$ M), 2  $\mu$ l reverse primer (10  $\mu$ M), 1  $\mu$ l template DNA taken from the first PCR, 1  $\mu$ l 50x Advantage 2 DNA polymerase and 37  $\mu$ l MilliQ water to bring the total reaction volume to 50  $\mu$ l. The PCR program was based on a combination of the manufacturer's instructions and the annealing temperature from Leus et al.(2006). The PCR program started by 1 min at 95°C followed by 35 cycles of 30 sec 95°C, 1 min 60°C and 1 min 68°C, after those cycles the temperature was set at 68°C for one minute.

For the amplification of the fragments with the MV primers a direct PCR was used in combination with the Advantage DNA polymerase. This was only used for the PCR where the fragments were going to be used for cloning in combination with the T Easy vector system. The PCR reaction was done in a total volume of 50  $\mu$ l. the reaction mixture consisted of 5  $\mu$ l 10x Advantage buffer, 2  $\mu$ l dNTP's (10 mM), 2  $\mu$ l forward primer (10  $\mu$ M), 2  $\mu$ l reverse primer (10  $\mu$ M), 1  $\mu$ l template DNA, 1  $\mu$ l 50x Advantage 2 DNA polymerase and 37  $\mu$ l MilliQ water to bring the total reaction volume to 50  $\mu$ l. The PCR program was based on a combination of the manufacturer's instructions and the annealing temperature from the primers as determined by BLAST primerdesign. The PCR program started by 1 min at 95°C followed by 35 cycles of 30 sec 95°C, 1 min 56°C and 1 min 68°C, after those cycles the temperature was set at 68°C for one minute.

## Ligation and cloning

The PCR product obtained using the Phusion and the Advantage DNA polymerase was visualized under UV light after electrophoresis (1.5 % agarose). The DNA was purified using the Zymoclean gel DNA recovery kit. The purified DNA was ligated in the pGEM-T Easy Vector system (Promega) (Figure 2) according to the manufacturer's instructions. The plasmid was cloned into XL10-Gold ultracompetent cells (Agilent technologies). The cells were cultured overnight at 37°C on LB plates containing ampicillin, X-gal and IPTG. Positive colonies were picked from the plates and grown on liquid LB medium containing ampicillin, simultaneously a PCR was performed on the colonies as described above to select colonies for sequencing.

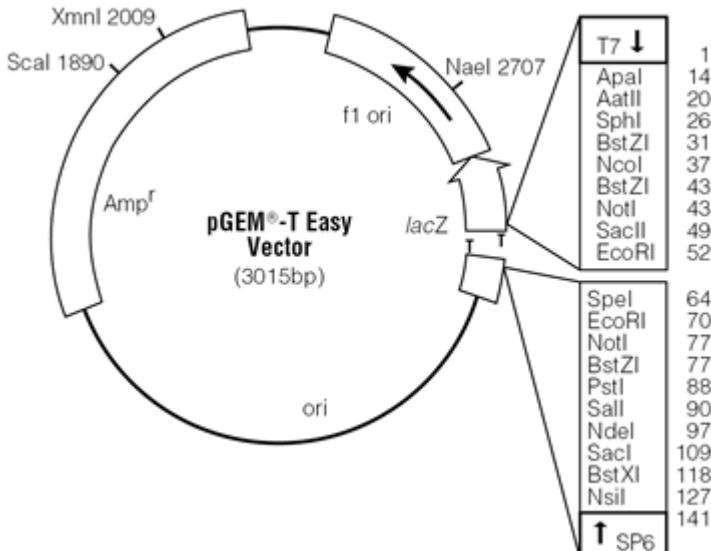


Figure 2: pGEM-T easy vector

## Sequencing and bioinformatics

The plasmids were isolated from the XL10-Gold ultracompetent cells using the Qiagen plasmid isolation buffer set (Qiagen). The kit was used according to manufacturer's instructions. 5  $\mu$ l of M13 reverse primer (10  $\mu$ M 5'-CAGGAAACAGCTATGAC-3') was added to 5  $\mu$ l of isolated plasmid. This mixture was sent for sequencing to GATC sequencing services using the Sanger sequencing technique. All the obtained sequences were aligned. The software used in this project was MEGA 5.2 and the ClustalW algorithm was used to create the alignments. The ClustalW method is a high power aligning algorithm that can align short sequences very accurate (Larkin et al. 2007). Both for the pair wise alignments as

well as for the multi alignments a gap opening penalty of 5 was used and a gap expansion penalty of 6.66 was used. This created an alignment that seemed correct after visual inspection.

Besides the sequences obtained from the samples also two sequences from the BLAST database were included in the alignment, the Leus R-P isolate (GenBank: DQ 139433.1) and the Leus R-E isolate (GenBank: DQ 139413.1). These sequences cover both ITSs and a small part of the rDNA. The parts that align with the two sequences from the BLAST database can be BLASTed themselves. This is to see what isolate from the database fits best to the sequence from the samples.

## ***In vitro* culture**

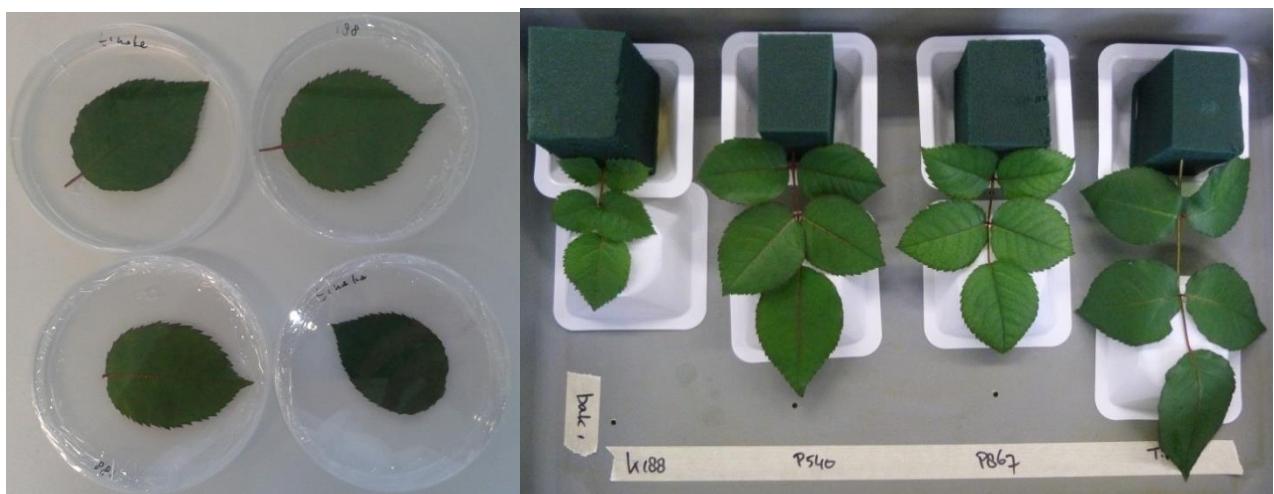
Eight plant cuttings obtained from older *in vitro* plants were planted in a 8 x 14 cm plastic containers filled with 100 ml rose maintenance medium (Appendix 1) and placed in a climate cell for four weeks. The climate cell was set at 24°C with 45% rh, there was 33 $\mu$ M/s<sup>-1</sup>/m<sup>2</sup> of light for 16 hours with an eight hour dark period. After four weeks, part of the plant population was cut again to maintain the population and part was transferred to new medium and infected after four weeks.

The four week old plants destined to be infected were placed in fresh rose maintenance medium. After four days, the infection occurred by gently rubbing a leaf from the Weiss cabinet that was infected with powdery mildew from the Melle isolate over the *in vitro* plants. After the infection the containers were covered with AirPore tape sheets (Qaigen) to reduce humidity in the containers. The infected plants were placed in another climate cell. This climate cell was set at 21°C with 50% relative humidity, 33 $\mu$ M/s<sup>-1</sup>/m<sup>2</sup> light for 16 hours and an eight hour dark period.

To deal with unwanted fungal infections we used the standard rose maintenance medium with added nystatin (25 mg/l) and miconazolnitrate (25 mg/l). The fungicides were filter sterilized and added after autoclaving the medium to prevent breakdown of the compounds. It is not known if the fungicides in the medium have any influence on the growth of the powdery mildew on the plant. It is not the intention to use fungicides in the medium more than one time. It will only be used to get an initial clean culture.

## **Detached leaf assay**

*P. pannosa* spores were isolated from infected leaves. Six infected leaflets were put in 20 ml tap water and vortexed for five minutes. A spore count was performed using a Fuchs-Rosenthal counting chamber. This spore suspension was used for maximum one hour because the spores die rapidly when they are in contact with water longer (Gubler and Koike 2009). Four genotypes were selected from the K5 rose population. One genotype was known to be susceptible to powdery mildew and one genotype was known to be less susceptible.



**Figure 3: detached leaf assay setup. Left: Petri dishes with MS medium and leaflets (Tineke, K188, P540 and P867). Right: leaves in floral foam (Tineke, K188, P540 and P867).**

From the other two genotypes, it was unknown whether they were susceptible or not. From each genotype, five leaflet leaves were picked. Then, they were placed into floral foam soaked with tap water and placed on a small plastic plate. Another plastic plate was turned upside down to support the leaf (, right). The leaves were each sprayed once with the *P. pannosa* spore suspension using a hairdressers spray (Figure 4). The leaves were placed in a climate chamber set at 21°C, the first hour the box was uncovered to allow the leaves to dry after that the box was covered with a transparent lid. After the lid was placed back on the boxes the relative humidity remained at 50%. The leaves were scored every day and when necessary tap water was used to keep the floral foam wet.



**Figure 4: the hairdressers spray used to apply the spore suspension on the detached leaves.**

With the same genotypes a detached leaf assay was conducted in a different form. The individual leaflets were placed on a Petri dish with medium (Figure 3, left). The medium consisted of 4.4 g of MS salts+ vitamins and 8 g of daishin agar per litre, no carbon source was added since the leaflets are still capable of photosynthesis and can thereby provide their own sugars. The leaflets were sterilised by submerging them into 1.5% hypochlorite for 10 minutes. After the sterilisation the leaflets were rinsed in sterile water to eliminate any effect of the hypochlorite on the growth of the powdery mildew. One leaflet was placed on each Petri dish and inoculated using an inoculation loop. The inoculation loop was brushed over an infected leaf before stroking it over the uninfected leaf four times. The sealed Petri dishes were placed in a climate chamber set at 21°C with 50% relative humidity, 33  $\mu\text{M}/\text{s}^1/\text{m}^2$  light for 16 hours and an eight hour dark period. Progress of the infection was checked on a daily basis.

## Results

### DNA isolation

DNA was successfully isolated with the Chelex method, and it was possible to perform a successful PCR using that DNA. There is however an autoclave step in the protocol in which in half of the cases, the sample evaporates in the autoclave, and is thereby destroyed. The Wizard Magnetic DNA purification system for food (Promega) provided a sufficient amount of DNA for the analysis. On average, 3 mg of DNA was extracted per sample of 100 $\mu$ l (30 ng/  $\mu$ l) which proved to be sufficient for further experiments although the 260/280 ratio of 1.3 indicated some protein contamination. According to Nanodrop a 260/280 ratio of 1.8 is considered pure and anything lower indicates protein or reagent contamination. This contamination did not have any influence on the follow up PCRs.

### PCR and sequencing

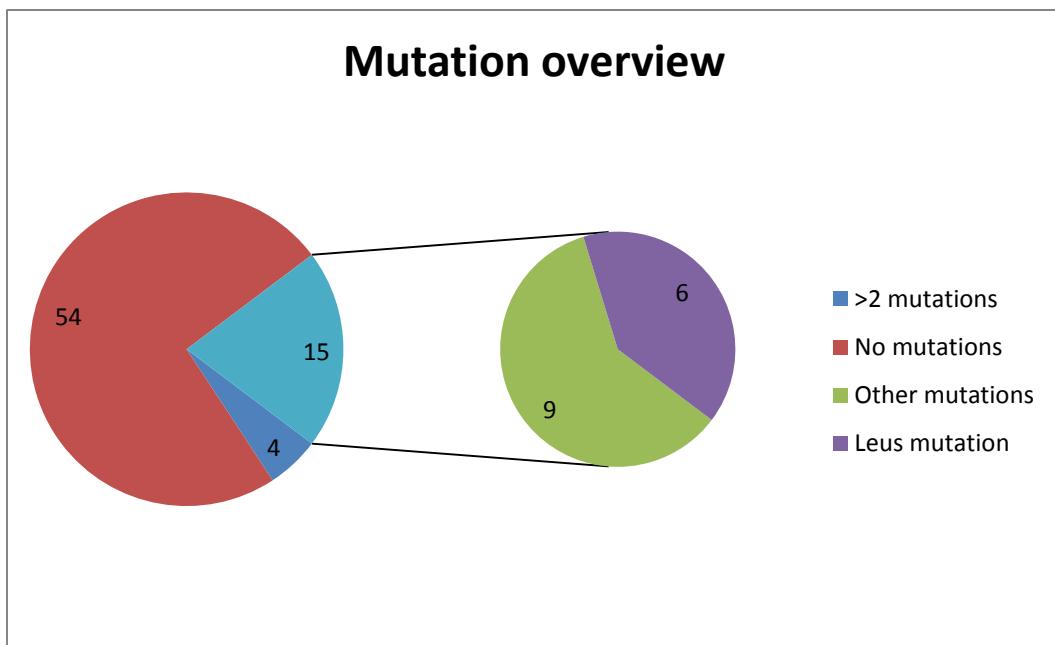
PCRs amplicons were obtained with two of the four primer sets with the powdery mildew samples from Hannover, Melle and Wageningen. Amplicons with a length of 430 bp were obtained with both the MV primers and the primers from Leus et al. (2006) which was according to our expectations. No size differences were observed that could help in discrimination of isolates/pathotypes, so sequencing is required. No amplification was obtained with the podo primers. The PCRs with the samples from De Lier and De Kwakel were only successful with the Leus primer set.

For sequencing, all the PCR products were ligated into vectors. The vectors were all cloned into competent cells. Plasmid DNA was isolated from all the picked colonies and it was sent for sequencing. Out of the 114 samples sent for sequencing, 73 showed >99 % of homology with the *P. pannosa* DNA sequence (GenBank: KF571742.1). The 74 positive samples are the 39 positive samples from the Leus primers and the 34 positive samples from the MV primers (Table 1). Also the E-value was in all cases  $<10^{-10}$  and sometimes even 0 indicating a very good to perfect match in the sequence. The other samples indicated as negative in table 1 showed homology with a wide variety of different fungal DNA sequence due to the lack of specificity of the primers. There were also vectors without inserts which had escaped the selection.

**Table 1: specification of the sequence result.**

Primer	Leus		MV		Sum
	Pos	Neg	Pos	Neg	
Hannover	12	4	11	1	28
Melle	13	2	11	4	30
Wageningen	7	11	12	3	33
Lex+ (De Lier)	5	7	0	0	12
Terra Nigra (De Kwakel)	2	9	0	0	11
Sum	39	33	34	8	114

From the 73 sequences that were identified as *P. pannosa* by BLAST 54 were identical to each other, 15 showed one or two basepairs difference compared to the 54 non mutated and 4 showed a mutation percentage up to 25%. The 15 samples that showed a small mutation were split into two groups: the Leus mutation and other mutations (Figure 5). In the article were this research is based on Leen Leus described and A to G transition on position 170 in ITS1 (Leus et. al. 2006). This mutation is found in 6 out of the 74 samples.



**Figure 5: Overview from the mutations found in the ITS sequences**

The four samples with the high mutation rate are all identified as *P. pannosa* by the BLAST database the E-value was however higher and around 0.001 where with the other samples the E-value was  $<10^{-23}$  or even 0. The four samples with the high mutation percentage were all unique in their mutations and occurred from different isolates. Two of the four were found in the Wageningen isolate, one in the Melle isolate and one in the Hannover isolate.

The six samples with the Leus mutation all originated from the Wageningen isolate. The Leus mutation was not found in the Melle isolate. This is remarkable since one of the in vitro isolates that was used to start the Melle isolate contained the Leus mutation.

From the samples with other mutations two different samples show an identical double mutation. And A to C transition was found on position 114 in ITS2 and an T to G transition was found on position 134 in ITS2. One sample with this mutation originated from the Wageningen isolate and the other sample originated from the Melle isolate. The DNA was obtained through independent PCRs both with the MV primerset.

The other non-Leus mutations were all unique and were all spread over the different isolates and amplified with the different primersets. There was no isolate or primerset that had more or less mutations.

### ***In vitro* culture**

The problem with water covering the walls of the containers which dripped on the powdery mildew and thereby potentially killing it encountered in the *in vitro* culture in earlier research was solved by covering the containers with Airpore tape sheets (Qiagen). The containers that are covered with the tape do not show condensation on the walls while in the containers covered with standard lids the moisture is clearly present. The decrease in humidity in the containers resulted also in the dehydration of the medium which now needs replacement after two weeks instead of the usual four weeks.

For the first inoculation mildew from the Melle isolate in the Weiss cabinets was used. The plants were grown on medium without fungicides resulted in three plants infected with powdery mildew but also massive infection in the medium occurred. The powdery mildew infection in the *in vitro* plants was visible 11 days post infection (dpi). A second attempt to inoculate the *in vitro* plants with powdery mildew with fungicides in the medium had a similar infection pattern for the powdery mildew. Unfortunately the fungicides in the medium didn't stop all the unwanted growth on the medium, it did not even postpone heavy infections long enough to transfer an infection to a new container with new plants. Also the

amount of powdery mildew on the roses was less than expected. Only six per cent of the plants had a visible infection.

One infection on an *in vitro* plant was picked up from that *in vitro* plant and transferred to a new *in vitro* plantlet in a new container. In this new container the powdery mildew grew as well. The powdery mildew in the old container continued to grow without any of the plants yellowing or dying. This was however in a container that was covered with a normal lid and had condensation on the wall, an environmental factor we had considered lethal for the fungus based on literature.

## Detached leaf assay

A spore concentration of  $10^3$ - $10^4$  spores/ml is needed for a good infection according to Remmelt Groenwold. Using our spore collection method described in the materials and methods section of this report we achieved to get a concentration of  $10^4$  spores/ml which is in theory enough to perform a successful infection. In setup I, each detached leaf in foam was sprayed with the suspension while present in the tray, the trays were closed and placed in the climate chamber set at 21°C with 50% relative humidity,  $33\mu\text{M}/\text{s}^1/\text{m}^2$  light for 16 hours and an eight hour dark period. After fifteen days, we did not observe any visible infection but the leaves started to die, therefore we decided to terminate the experiment.

The detached leaf assay using the MS medium and the leaflets in Petri dishes (setup II) did not give any results. After fifteen days, the leaflets dried out and there was no visible *P. pannosa* infection. The leaflets all had been inoculated since tiny visible amounts of mycelium were left behind with the inoculation, this mycelium that was manually brought on the leafs disappeared over time.

## Discussion and conclusions

### DNA isolation

The DNA isolation using Chelex is a very simple and quick procedure but presents a high risk of losing the sample through evaporation in the autoclave. This makes it an unpractical DNA isolation method. The magnetic beads DNA isolation procedure gives enough yield for the follow up PCR and also has a simple and quick protocol. This makes it an ideal method to isolate the powdery mildew DNA. Although the purity of the DNA is not optimal, it is still good enough to perform a PCR and therefore the magnetic beads protocol was used as our preferred DNA isolation protocol.

### PCR and sequencing

Based on the gel electrophoresis results from the PCR with the four primer sets (Leus primer set, MV primer set, Podo 1 primer set and Podo 2 primer set), we can conclude that the Podo 1 and 2 primer sets did not show bands and therefore are not able to amplify powdery mildew DNA at the used conditions. The Leus primer set is able to amplify not only powdery mildew DNA but also DNA from a wide range of other fungi as can be deduced from the sequencing data. This result is expected but it decreases the chance that one would amplify powdery mildew in the PCR thus to get the same number of amplicons with the MV primers and the Leus primers one has to do more PCRs with the Leus primers. During one experiment the Leus primer set also amplified rose DNA but those results could not be replicated. The MV primer set is able to amplify powdery mildew DNA of have not found any amplicons from other fungal species it is however not clear if the MV primers can amplify all the powdery mildew isolates in existence. The specificity of the MV primers can be decreased by lowering the annealing temperature in the PCR reaction this will make sure all powdery mildew isolates are picked up but it will also dramatically increase the chance of amplifying DNA from other fungal species. The Leus primer set is ideal for analysing new found powdery mildew sources due to the wide range of the primers. The MV primer set can be used to monitor powdery mildew strains that are already analysed but need monitoring to see if there is contamination from other strains in a single strain culture.

Based on the ITS sequence we can divide our samples in three groups containing two or more samples and four individual samples. The three groups consist of the non-mutated samples, the samples with the Leus mutation and the third group is the group with the double mutation consisting of two samples. All

groups have representatives of at least two independent PCRs. This all leads to the conclusion that there are at least three different isolates in the samples taken from the five locations.

The four samples that do not fit in any of the groups, do not have anything in common with each other and have a lower similarity to powdery mildew sequences from the BLAST database cannot be called different isolates. There is a chance that the sequences found in this group are the result of a PCR or sequence error. When the same sequences are obtained in a new PCR then it is possible to call members of this group separate isolates.

The isolate with the Leus mutation that should have been in the Melle isolate has not been found. With the Melle isolate consisting of a mix of at least three isolates from which two were different for sure it is possible that the isolate with the Leus mutation was not in the sequence pool by chance. With 24 ITS sequences recovered from the Melle isolate the chance of not amplifying this isolate when it is evenly distributed in the Weiss cabinet is very small. A more likely conclusion is that the three isolates that make up the Melle isolate are not equally represented in the Weiss cabinet. This can be the result of unequal inoculation or due to difference in vigour between the three different isolates.

Leen Leus reported a different host range for the isolates carrying the Leus mutation, these isolates were able to infect not only rose but also *Prunus* (Leus et. al 2009). It is impossible to determine the host range of the isolates with the double mutation based on the ITS sequence.

With the possible difference in vigour between the isolates it is better to do the disease test with the three isolates separately. By using the separate isolates you are sure that you prevent one isolate outgrowing another. When one isolate outgrows another isolate the resistance against the isolate that has been expelled cannot be measured thereby you can end up with a plant resistant against two of the three isolates without knowing that.

Separation of the isolate can be done using the so called eyelash method (Coyier 1973). This method uses an eyelash to pick up an individual conidium with the aid of a microscope. The conidium is then placed on an *in vitro* plant so it can develop further there.

## ***In vitro* culture**

Due to the amount of infections in the medium and the little growth on the *in vitro* plants, we might have to conclude that it is not feasible to culture *P. pannosa* on *in vitro* roses with our methods. There might be a possibility to add even more antibiotics and fungicides to the medium but that might harm the plants and also inhibit the growth of powdery mildew. The effects on the powdery mildew on the plants of antibiotics and fungicides in medium that supports plants with powdery mildew are unknown. Furthermore the fungus needs to be transferred very often because of the tape sheet that covers the top of the container. Because of the permeability of the tape sheets for water vapour the medium dries out quickly. This makes the medium dry out completely in about three weeks. Other experiments done by Iris show that the *in vitro* roses suffer a lot more from the powdery mildew infection than adult plants in the Weiss cabinet or in the greenhouse. In *in vitro* culture plants die from the infection which is normally non-lethal. The very last results however, indicate that it is possible to maintain powdery mildew *in vitro* since we had one *in vitro* plant that had a powdery mildew infection which sustained itself for more than one week. It is however a process that right now relies very much on luck. Further experiments have to be done to optimize the process. Some positive results have been obtained by Iris. With the removal of the extra iron from the medium extra growth was seen. The increase in powdery mildew growth is due to the increase in phosphate availability. The iron in the medium binds phosphate and makes it thereby less available. With the removal of the iron more phosphate is available and the powdery mildew uses that (Sigma-Aldrig 2014).

The liquid water on the sides and lid of the container did appear to be a problem. All the experiments done by Iris were done using the regular plastic lids on the containers and not the tape sheets.

Apparently the pores do not come into contact with the liquid water and the inability to grow powdery mildew on *in vitro* plants earlier had other reasons. One reason that could be an explanation for the lack of vigour in the powdery mildew which was also seen in the Weiss cabinets when the fungus was just introduced to its new environment.

## Detached leaf assay

The results showed that it was not possible to perform a detached leaf assay using the methods described above. The fact that we obtained no results at all while we were using susceptible genotypes under optimal conditions can be blamed on the fact that a sulphur treatment was applied overnight in the greenhouse to the plants. This method is used to prevent powdery mildew infections, and we did not realize that the sulphur evaporation was going on in the greenhouse where and while we collected the leaves for our assays.

It is however worthwhile to investigate this method further since it will dramatically enlarge the scale on which resistant tests can be done when the test is operational and can give reliable results.

## Future research

### PCR and sequencing

At the moment, the sequencing step in the protocol takes most of the time due to the making of the plasmids and the cloning. It would be nice to have a restriction enzyme that could digest the PCR product in a unique pattern for each of the three groups. This would make identifying the different groups a lot quicker and cheaper. There is however the risk with this method that one would miss new groups but for monitoring the purity of isolates this could be a good method.

### *In vitro* culture of powdery mildew

The very few good results we obtained by culturing *P. pannosa* on *in vitro* roses indicates that a lot of optimisation has to be done to make this process more efficient and with a higher success rate. This can be done by making variation in medium, climate conditions and *in vitro* rose genotypes. The powdery mildew that grows on the rose should also be analysed to see in which of the three groups it belongs or even whether it is a new group in the tree.

### Disease tests

Despite the marginal results we had in tissue culture with powdery mildew, I still believe it is possible to maintain *P. pannosa* *in vitro* but a lot of work has to be done to optimize the growth conditions. When those conditions will be known, it should be possible to perform a detached leaf assay under the same conditions with hopefully positive results. Obviously, the detached leaf assay should be performed again with leaves that have not been exposed to sulphur or treated in any other way for fungal control. The results of those tests should however in the beginning always be checked against disease tests in normal rose populations with adult plants to confirm the results.

In case the disease tests do not work with the current conditions adjustments could be made to the light. Both in intensity and spectrum it can have a large effect on powdery mildew (Quinn and Powel 1981). The effect of moisture and relative humidity is also a factor that is well discussed within this report and very important to keep in mind when searching for the optimal growth conditions for powdery mildew

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## Appendix 1: Media used

1 litre LB agar medium

- 15 g agar
- 10 g peptone
- 10 g NaCl
- 5 g Bacto yeast extract

After autoclaving, 20 minutes at 121°C and 1 bar

- X-gal (500x): 2ml
- Ampicillin (500x): 2ml
- IPTG (1000x): 1ml

1 litre rose maintenance medium

- 4.4 g Murashige and Skoog medium plus vitamins
- 96 mg Fe-EDDHA/L
- 30 g glucose
- 1 ml BAP
- 8 g daishin agar

pH set at 5.8

1 litre MS agar medium

- 4.4 g Murashige and Skoog medium plus vitamins
- 8 g daishin agar

pH set at 5.8

## Appendix 2: Protocols

### Detached leaf assay floral foam method

- Stick one 5 leaflet leafs in a piece of tap water soaked florists foam.
- The floral foam is placed on plastic dishes and the leaves rest on an identical plastic dish turned upside down. Everything has to be in closable trays with a transparent covers.
- Prepare the spore suspension:
  - o Take six powdery mildew infected leaflets and vortex for 5 minutes in 20 ml tap water.
  - o Check the spore count using a Fuchs-Rosenthal counting chamber, the spore concentration should be between  $10^3$  and  $10^4$  spores per ml.
  - o Use the suspension within one hour.
- Spray the spore suspension over the leaves
- Let the leaves dry in the open air for one hour, then close the trays and place in a growth chamber set at 21°C

### Detached leaf assay MS medium method

- Prepare the medium.
  - o 4.4 g MS + vitamins medium
  - o 8 g agar
  - o 1 l MilliQ water
  - o Autoclave
- Pour the medium in Petri dishes
- Place one uninfected rose leaflet in each Petri dish and make sure it touches the medium.
- Use an inoculation loop to scrape some mycelium of an infected leaf and brush over the infected leaf to inoculate.
- Seal the plates and place in a growth chamber set at 21°C.