Fungicide resistance against fluopyram in *Alternaria solani*

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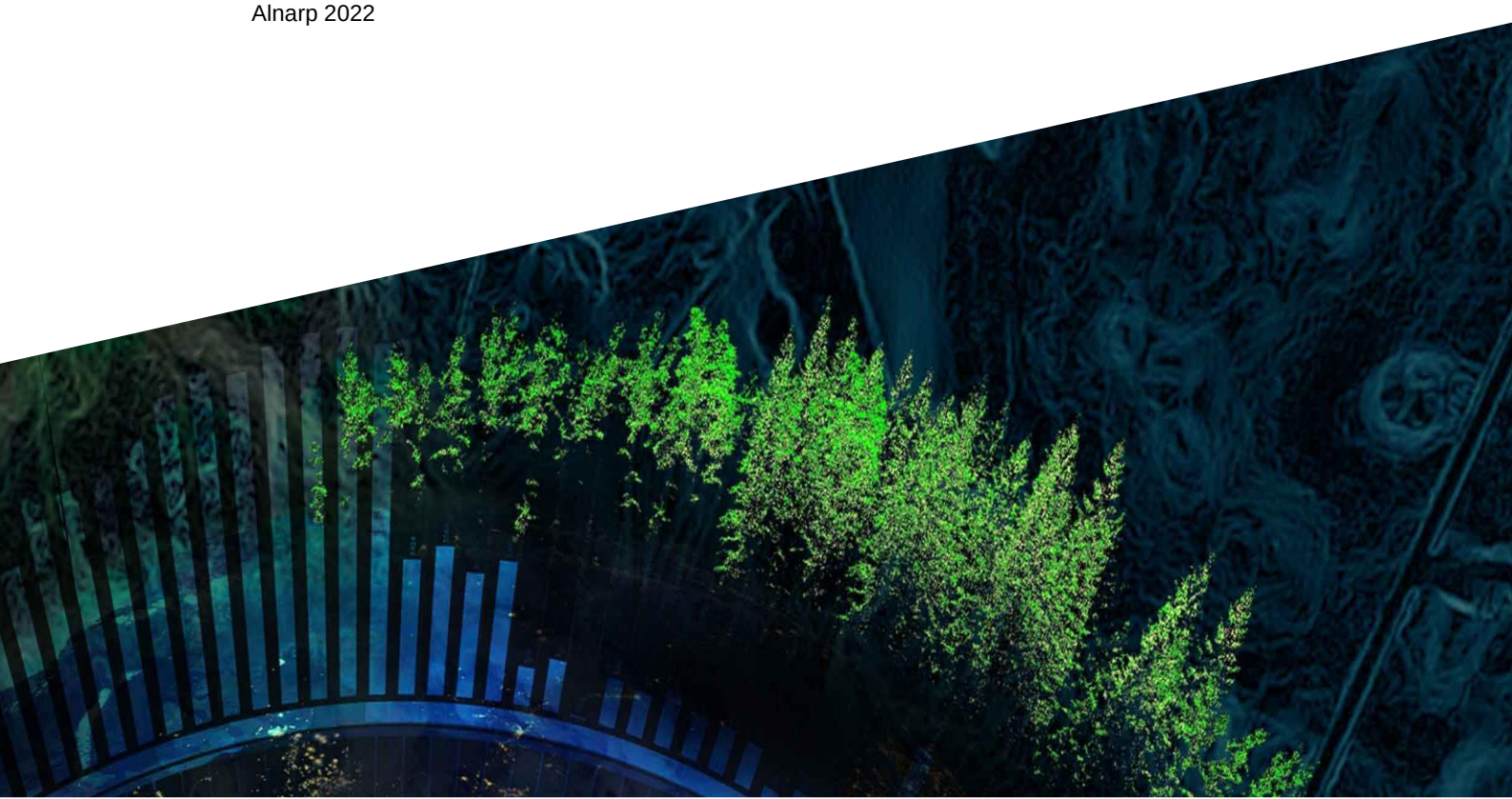

Fungicide Resistance against fluopyram in *Alternaria Solani*

Fungicidresistans mot fluopyram hos Alternaria solani

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Abstract

Early blight in potato production can be devastating and to minimize crop damage the use of fungicides has been the key. New fungicides called the SDHIs have been used against the early blight pathogen, *Alternaria Solani* in potato fields to reduce yield losses. However, repeated fungicide applications run the risk of fungicide resistance development in the fungal population, through the selection of mutations in the genes that are targeted by the fungicides. Mutations that confer a loss of sensitivity to the fungicide (i.e., fungicide resistance) will be selected for in fungal populations where there is a significant selection pressure, through for example, the repeated use of one class of fungicides. To investigate whether *A. solani* was becoming insensitive to the new SDHI fungicide fluopyram, a growth study was conducted where 20 different strains of *A.solani* were grown on solid medium with five different concentrations to see if some of the strains were less susceptible than others. It was difficult to draw in depth conclusions from this growth study, however some samples showed less sensitivity to the fluopyram. A DNA analysis of the fluopyram target gene was also performed to try to determine if there were any mutations associated with resistance present. However, due to problems with the PCR and sequencing experiments it was not possible to make any firm conclusions from this.

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Introduction

Alternaria solani

Early blight disease caused by the fungus *Alternaria solani* is a worldwide problem especially in potato (*Solanum tuberusum L.*) cultivation (Franc & Christ, 2001). It is categorized as a foliar pathogen that is difficult to control (Olanya et al., 2009) and can lead to devastating results, including decreased yield, if not treated (Price *et al.*, 2015). The syptoms of early blight occur first on the oldest foliage where it develops a characteristic brown to black lesions that are restricted by the leaf veins. Many things can reduce or increase the early blight outbreak such as temperature, moisture the crops age and maturity of the tubers (Palm & Rotem, 1997). The early blight increases most rapidly when tuber initiation has occurred and that can lead to destroyed foliage as well as reduced yield (Pscheidt and Stevenson 1986; Shtienberg et al. 1996).
According to VanDer Waals et al (2001), the yield losses can be between 20-50%. In Denmark they also did a study on the starch content in potato from 2010 to 2014 their results showed that the starch content increased between 7-20% in the sprayed fields compared to the unsprayed fields (Nielsen, 2015).

The commercial cultivars of potato are mostly susceptible to the disease (Christ, 1991), thus it was suggested that the best way to decrease the damage is to apply protective fungicides from early in the cultivation until the plant starts to dry (Pscheidt & Stevenson, 1988). However, due to genetic variation of pathogen population some individuals will be less sensitive to the fungicide (Lewontin, 1974). Natural selection, which allows for adaptation to the environment, e.g., under selection pressure such as repeated contact with a toxic substance such as a fungicide, will lead to an increase in the prevalence of isolates of the pathogen that are resistant to the fungicide preparation used (Dekker, 1995; Avenot & Michailides, 2010), which would increase both frequency of fungicide use and burden the ecosystem.

Propulse

Propulse SE 250 is a preparation used against *A.solani* in potato cultures but it is also used against other fungi attacks in other cultivars such as oat, wheat and barley (Bayer crop science, n.d.). The active ingredients are the SDHI fluopyram which inhibits the cellular respiration in the fungi and protiokonazol that inhibits the fungi build-up of the cellmembrane. The fluopyram together with the protiokonazol makes for a preparation that works both preventively and minimize further infection. When applied the preparation are evenly spread throughout the vascular system in the leaves and can therefore be used before and when a fungi infection already has occurred.

SDHI

Succinate dehydrogenase inhibitors (SDHIs) are included in a variation of fungicides and disturb the function of the enzyme succinate dehydrogenase (Sdh). Within the mitochondria complex II the Sdh enzyme suppresses the process of cellular respiration and binds to one of the subunits SdhB, SdhC, SdhD (Mallik et al., 2014; Sierotzki & Scalliet, 2013; Pasche et al., 2005). The Sdh enzyme contains four subunits which are called SdhA, SdhB, SdhC and SdhD. There are different compounds that are classified as SDHIs, i.e. fluopyram, boscalid and isopyrazam. In 1960 the early SDHI fungicides that were based on carboxin were introduced to the agricultural setting, their main targets was against the diseases caused by basidiomycetes and Rhizoctonia (Zhang et al., 2009; Yanase et al., 2007; Ulrich and Mathre, 1972). The SDHIs that were introduced later i.e. boscalid and fluopyram had a broader spectrum of diseases and worked on more crops (Stammler et al., 2007; Yanase et al., 2007). When boscalid was established in agricultural practices it minimized the damage of early blight cause by fungal pathogens (Pasche & Gudmestad, 2008). However, use of SDHI fungicides over time led to development of resistance among various pathogens.

Pathogens such as *Alternaria alternata* and *Botrytis cinerea* among others are included in a group that has high risk of developing resistance to fungicides (Sierotzki & Scalliet, 2013). Resistance to SDHI fungicides that has occurred in other fungal plant pathogens were due to exchanges in amino acids in the subunits B (SdhB), C (SdhC), D (SdhD) (Mallik et al., 2014). Resistance to the SDHI fungicide boscalid primarily revealed mutations in SdhB, SdhC and SdhD and not in the SdhA (Avenot & Michailides, 2010; Avenot et al., 2009; Avenot et al., 2008).

When SDHIs are used too frequently and at inappropriate concentrations they can potentially cause resistance in the pathogens against the SDHIs (Avenot et al., 2009, Bardas et al., 2010; Miyamoto et al., 2010; Avenot et al., 2012; Dekker, 1995). According to Avenot & Michailides (2010) shortly after the SDHI boscalid was released onto the commercial stage a limited number of pathogens started to show signs of resistance. Fungicide resistance has already been confirmed, in the United States Wharton et al (2012) detected that *A.solani* showed less susceptibility to the use of the fungicide boscalid. This problem is not only showed in the United States but also detected in Europe (Landschoot et al, 2017; Metz et al, 2019). In Sweden there has been detection of resistance in *A. solani* to the fungicide boscalid the conclusion was that prevention of early blight should not rely on boscalid at least not in south of Sweden (Mostafanezhad et al, 2021). Furthermore according to Odilbekov et al (2019) *A.solani* populations showed that during season the population changed as a response to the fungicide application.

Aim

To investigate if *A. solani* isolates collected in southern Sweden display reduced susceptibility to the SDHI fluopyram preparations and to see if the resistance can be shown as mutations in the DNA sequence, specifically in the fluopyram target, the *SdhB* gene.

Research questions

Growth

Does the fungus show different growth depending on the concentration of fluopyram?
Do the isolates collected in different years show signs of a different growth pattern depending on a longer exposure to fluopyram?
Has the population become more resistant to fluorpyram over time- i.e. will newer isolates show more resistance to fluorpyram than those collected longer ago?

DNA Analysis

Has a mutation occurred? Are the potential mutations the same as the ones identified in boscalid resistant isolates?
Are there any risks with continuous use of fluopyram?

Hypothesis

The fungal growth will decrease exponentially in relation to the increased concentration of fluopyram.
The fungicide resistance can be linked to a specific mutation in the DNA.

Materials & Methods

Growth

Alternaria solani cultures

A. solani isolates were collected from infected potato plants in field trials in the years 2018, 2019 and 2020. In each year three isolates were collected in the part of the field unsprayed with fungicides against early blight and three samples were collected from sprayed areas. For all the years (2018, 2019 & 2020) Narita and Propulse were used as fungicides in the sprayed areas. Additional two isolates collected in 2014, with no detection of mutations associated with fungicide resistance, were used as a reference.

Solid medium preparation

In five different bottles 1.92 g of potato dextrose agar and 3 g bacto agar were added together and filled up to 250 ml with MQ water. These ingredients were then mixed together and the medium was autoclaved for 20 min in 1.5 bar/Psi at 125℃. Afterwards the bottles were placed in the incubator at 60 ℃ for storage until use.
To prepare 40 mM stock solution 18.75 mg of chlortetracycline (CTC) were weighed and dissolved in 1 ml 70% EtOH and stored in the freezer for later use. CTC is a tetracycline antibiotic that is commonly used for minimizing bacteria growth.

Before use the bottles with growth medium were removed from the incubator one by one and the prepared CTC as well as fluopyram were added into the media. Fluopyram was added in different concentrations (Table 1). The stock solution had a concentration of 24 µg/ml. The bottles were stirred before 10 ml of the potato dextrose agar were transferred from the bottle to the Petri plates.

Table 1:The different amounts of fluopyram that were added to the different bottles of agar solution.

Bottle number	amount of stock Fluopyram solution added	final concentration of Fluopyram
1	—	0.00 µg/ml
2	208 µl	0.02 µg/ml
3	625 µl	0.06 µg/ml
4	1250 µl	0.12 µg/ml
5	10417 µl	1.0 µg/ml

Agar plate study

Mycelial cultures grown on solid medium were looked at through a stereo microscope to decide where to pick the best *A. solani* growth. All equipment was then moved into the laminar flow hood sterilised with both 70% Ethanol (EtOH) and 10% bleach. With a cork borer five plugs were made at the marked place in each colony. All the plugs were moved to media with different fluopyram concentrations (0.0, 0.02, 0.06, 0.12 and 1.0 µg/ml). Each plate was inoculated with a single agar plug. The plates were then sealed with parafilm and stored at room temperature. The diameter of the mycelia growing around the plug was then measured each day for 14 days.

Growth pattern was calculated on the k-value for each fluopyram concentration and sample.

Figure 1 is an example and shows all the measuring data from the different concentrations of fluopyram with sample A.S 193 of the A. solani. A trendline where inserted to be able to calculate the k-value, the k-values where later used in figure 2. In appendix 1 all the calculated k-values for each of the samples and concentrations can be found.

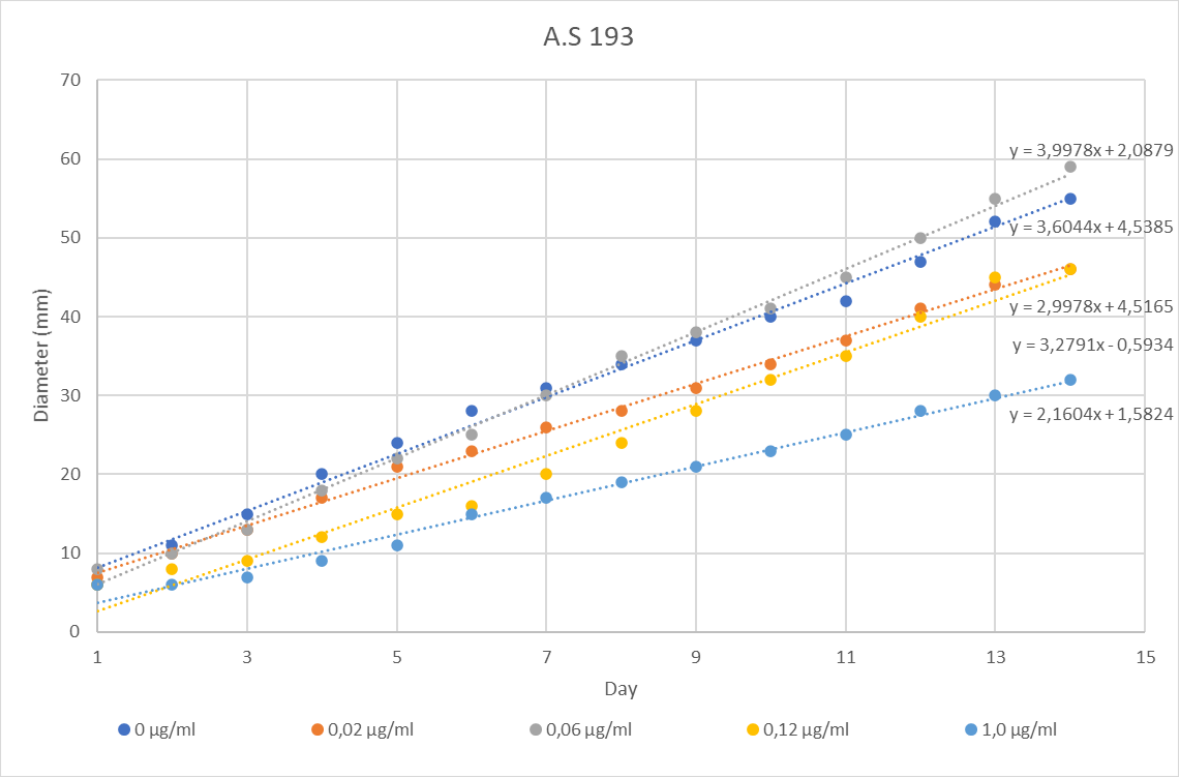


Figure 1: All the measuring data from the different concentrations of sample A.S 193 for each day that where measured. The k-value where then calculated for each of the concentration.

DNA Analysis

DNA extraction

Total genomic DNA was extracted from the different isolates with DNeasy Plant Mini kit from Qiagen according to the manufacturer's protocol. The DNA extraction was completed twice, once with freeze dried mycelia samples from *A. solani* isolates that were disrupted in an Eppendorf tube with a plastic pestle. The second time the isolates were grown in liquid media instead of agar plates and snap frozen in liquid nitrogen. Some cultures only grew once due to the extraction method and the vitality of the isolates.

Liquid medium

Table 2: Recipe for 1L liquid medium.

Chemicals	Amount (g)
$NaNO_3$	3
K_2HPO_4	1
$MgSO_4+7H_2O$	0.5
KCL	0.5
$FeSO_4+7H_2O$	0.01
Sucrose	30
Yeast extract	20

Nanodrop quantification

Nanodrop is an instrument that estimates the DNA concentration of a sample based on its absorbance at 260nm. Ratios of absorbance at 260nm to absorbance at 280nm and 230nm are used to estimate sample purity. Since DNA extraction was carried out twice, using two different methods, the samples with the best quality, i.e. both absorbance ratios closest to optimal (2.0) were chosen for further experiments.

PCR amplification

The polymerase chain reaction (PCR) was conducted using 5 µl Platinum SuperFi Buffer (5x), 2.5 µl dNTPs (2µM), 0.5 µl F-primer (10 µM), 0.5 µl R-primer (10µM) and 0.5 µl Platinum SuperFi polymerase (2U/µl). The DNA was mostly matched to 30 ng and the volume was made up to 25 µl with MQ water.
The primers used in this project were SdhB primers used in Mallik et al. (2014): SdhB-F 5´ATGGCCTCCATACGCGCTTT 3´and SdhB-R 5′ CTAGGTGAAGGCCATGCTCTT 3′.
Program: The first part is the denaturing stage at 95℃ in 2 minutes, it breaks the hydrogen bonds between the two strands of DNA. Second part is the annealing stage with 30 cycles at 95℃ 30 seconds, 60℃ 30 seconds and 72℃ 1 minute. This is where the primers connect to the single stranded DNA. The last part is the extending also called the elongation stage at 72℃ for 7 minutes where the Taq DNA polymerese enzyme adds DNA bases.

Gel electrophoresis

The gel-casting tray was assembled on a bench, 2 g of agarose standard was weighed and mixed with 1xTAE buffer which was poured up to the 200ml mark in a 250 ml beaker. The mixture was then moved to the microwave and heated until all the agarose dissolved. Afterwards 20 µl of GelRed, Nucleic Acid Stain (Biotium Inc.) in concentration 10000x was added. The mixture was then poured to the casting tray with combs and left to cool down and solidify. When the gel had set it was placed into the gel tank and 1 x TAE was poured over to about 2-3 mm above the gel. The combs were then gently removed. In the first well 5 µl of 1kb GeneRuler (Thermo Scentific) were added, afterwards 5 µl of each sample were mixed with 1 µl 6x DNA gel loading dye (Fermentas) and then added to a well. This procedure was repeated for all the samples. The gel was run for 30 min at 90 V before visualization in a UV-chamber.
For band excision a 2% gel was used, i.e. 4 g of agarose standard, 1xTAE buffer poured up to the 200 ml mark in a 250 ml beaker and 20µl of GelRed (Biotium Inc.).

Precipitation & Purification

First method was an ethanol precipitation using 70% ethanol and 3M NaAc. For both the gel extraction and the PCR purification the kit PureLink Quick Gel Extraction & PCR Purification Combo Kit from ThermoFisher was used, both times according to the manufacturer's protocol.

Results

Growth

The *A. solani* isolates were collected in potato field trials in years 2018, 2019 and 2020. We selected three isolates that were collected in unsprayed areas and three isolates that were collected in sprayed areas of the field for each year. The sprayed parts of the field were treated with Narita and Propulse commercial fungicides. Additionally, two isolates collected in 2014 and confirmed not to have mutations related to fungicide resistance were used as "wild type" reference isolates. All isolates were tested on each concentration which resulted in 100 agar plates, the growth diameters of these were all measured for 14 days (appendix 1), each isolate was studied in relation to the concentration of fluopyram (Figure 1).

All the samples in figure 2 shows a varied growth speed in correlation to the concentration of fluopyram. Addition of fluopyram has a noticeable effect on the growth of all the samples, the higher the fluopyram concentration the larger the growth reduction. Furthermore, the wild type samples have a higher growth rate reduction amongst the higher concentration of fluopyram than e.g. the untreated sample from 2019. Even if the untreated samples from 2020 and the wild types show similar growth rate in the 0.0 µg/ml, 0.02 µg/ml and 0.06 µg/ml concentration of fluopyram, their growth pattern diverge at the 0.12 µg/ml and the wild types show a higher sensitivity to the 1 µg/ml concentration of fluopyram.than the untreated sample from 2020. Treated samples from 2018 showed similar growth pattern as untreated samples from 2018 at 0.0 µg/ml of fluopyram however, the reaction the added fluopyram made the treated samples from 2018 show more sensitivity against fluopyram than the untreated. However, the untreated samples compared to the treated samples showed no distinct difference in the growth pattern in correlation to the concentration of fluopyram.

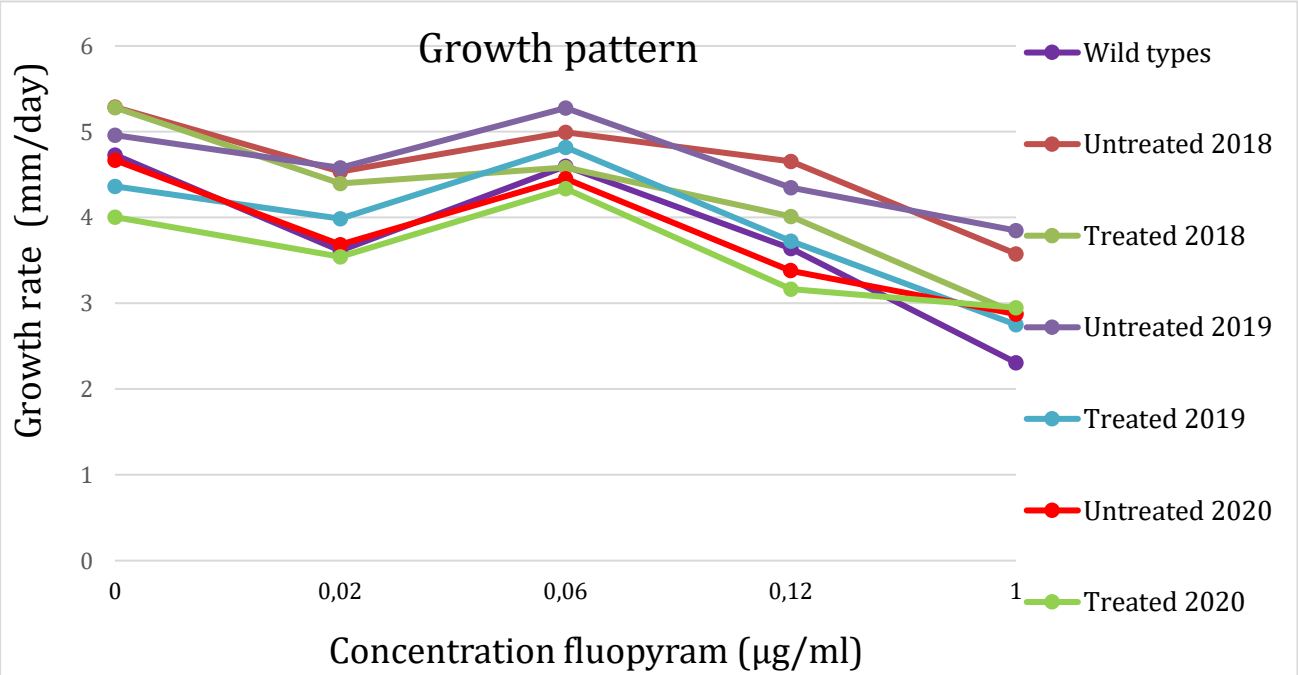


Figure 2: Growth pattern of the different Alternaria solani isolates at increasing concentrations of fluopyram. The data were categorized by the year the isolates were collected and whether they were collected in the fungicide treated (treated) or unsprayed (untreated)

The wild type samples showed a high reduction in growth within the concentration of 1 µg/ml of fluopyram (figure 3). Furthermore, the sensitivity against fluopyram has declined among the treated samples for each year. The untreated samples showed similar decreased sensitivity between the years of 2018 to 2019. However, the sensitivity of the untreated samples of 2020 increased from 2019.

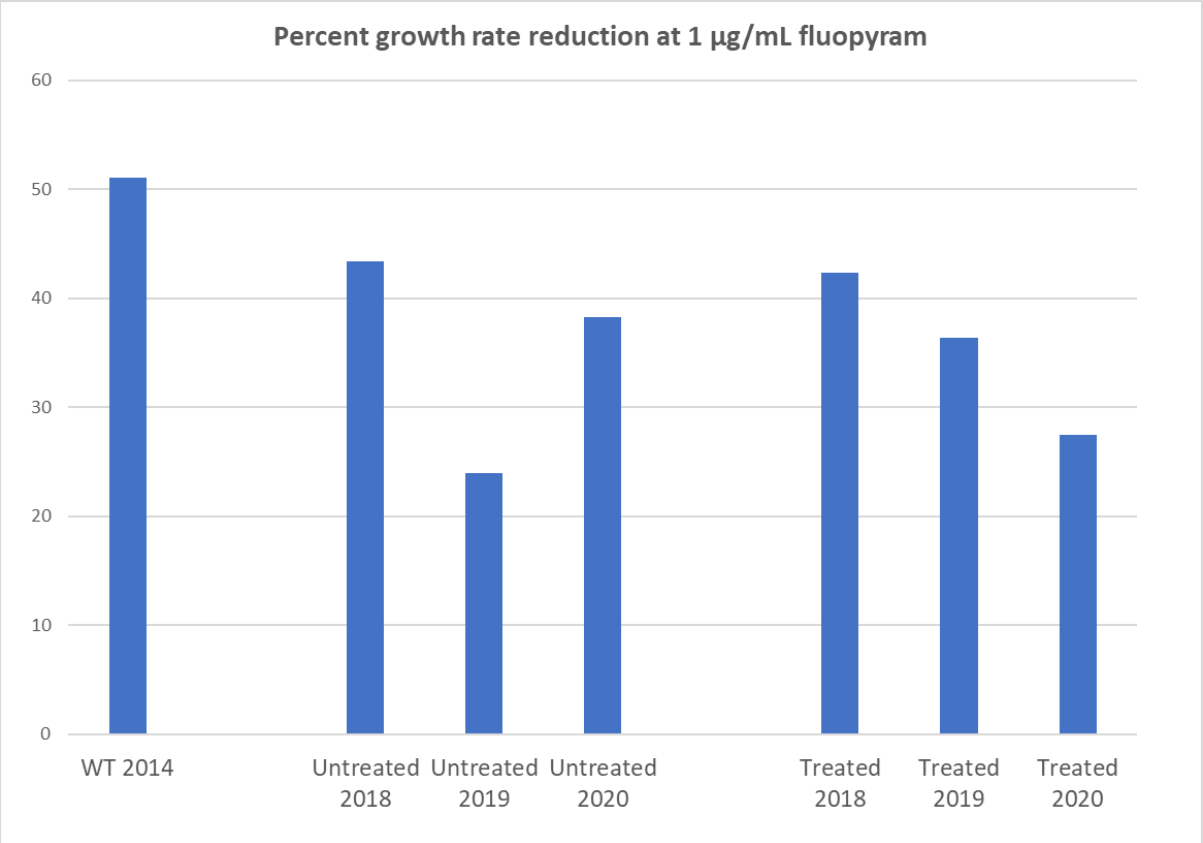


Figure 3:Growth reduction in percentage at 1 µg/ml of fluopyram. The data is categorized in the wild type samples, untreated samples and treated samples.

DNA analysis

DNA was extracted twice with different methods to see if the DNA quality did improve. The first DNA extraction was made on freeze dried mycelium. After the extraction the DNA quantity and quality was evaluated on a NanoDrop. Some of the samples were ethanol precipitated to increase the concentration and purity of the sample.

The first attempt with DNA extraction gave various concentrations as well as sample purity (Table 2). For the concentrations the values varied from the highest at 83.1 ng/µl to the lowest at 6.9 ng/µl. For some of the samples the 260 nm/230 nm absorbance ratio reached over the normal value (optimal 2.0) for example the sample of 3.4.10.2, A.S 41, A.S 190 and 3.3.10.3 all had a value of over 5.0. During the purification phase those that had values over 5.0

decreased to have values under 3.0 which were closer to the optimum (2.0). After the purification process the concentration did increase on all the samples that were purified, the samples N19.4.3 and N19.1.1 increased to a value over 100 ng/µl.

Table 3: The concentration and absorbance ratios of the DNA samples from the first DNA extraction. The red numbers are the result after the purification process.

SAMPLE NAME	CONCENTRATION [ng/µl]	260/280	260/230
N3.3.1.1	14.7	1.85	1.83
N20.1.2	16.1/32.7	1.74/1.88	1.46/1.16
N19.4.3	83.1/185.3	1.77/1.75	0.92/0.84
N20.1.3	13.6	2.08	2.76
N19.1.1	46.7/112.6	1.76/1.74	0.87/0.76
N20.2.1	9.3	1.67	1.32
N3.4.10.2	10.2	1.84	6.98
N19.1.3 A	13.7/25.1	1.69/1.75	2.45/1.44
N3.3.10.4	14.7/45.5	1.76/1.76	2.21/1.44
N20.1.4	8.6	1.67	6.17
A.S SBS	12.8/23.5	1.75/1.85	2.62/1.92
A.S 8	27.1/53.0	1.67/1.83	1.87/1.51
A.S 41	6.9/77.2	1.96/1.57	5.37/0.86
N3.2.1.1	15.9/90.3	16.9/1.54	1.02/0.85
N19.3.3	11.9/28.7	1.67/1.66	2.50/1.08
N3.4.1.5	32.0	1.77	0.96
N20.2.2	69.5	1.86	2.12
N19.3.1	72.8	1.76	0.94
N20.1.1	70.5	1.85	2.42
N3.2.10.5	9.0/16.6	1.83/1.81	4.91/1.85
N3.1.1.3	15.0	1.72	1.16

A.S 190	11.4/14.0	1.65/1.74	6.22/2.84
A.S 193	18.7/64.8	1.76/1.73	1.95/1.25
N3.3.10.3	11.2/14.5	1.69/1.69	7.50/1.95
N20.2.4	27.5/60.8	1.84/1.82	2.08/1.57

PCR reactions were conducted on all the DNA samples from Table 1 and afterwards run on a 1% agarose gel electrophoresis where all samples except two showed DNA bands.
once with freeze dried mycelia samples from *A. solani* isolates that were disrupted in an Eppendorf tube with a plastic pestle.

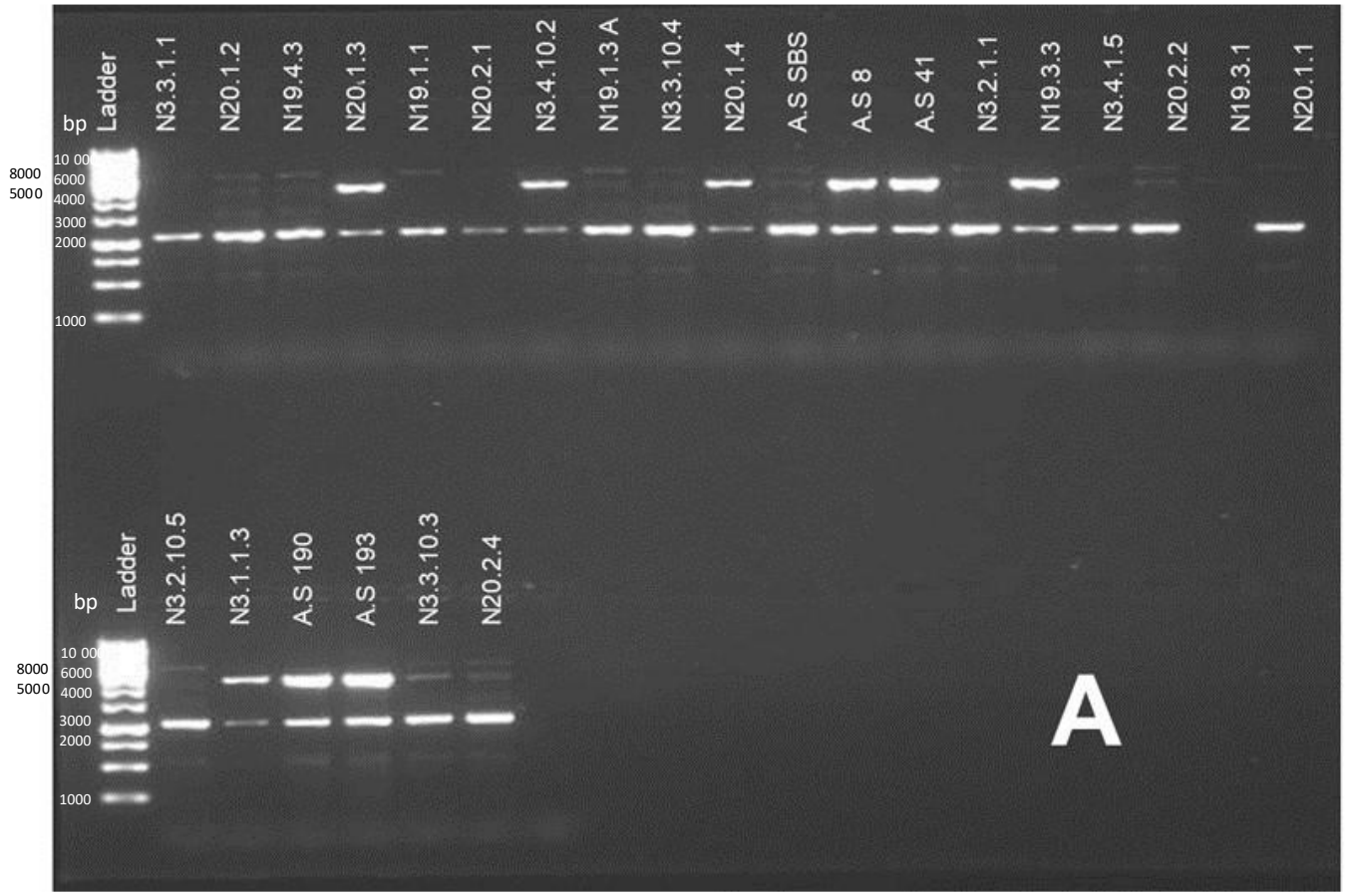
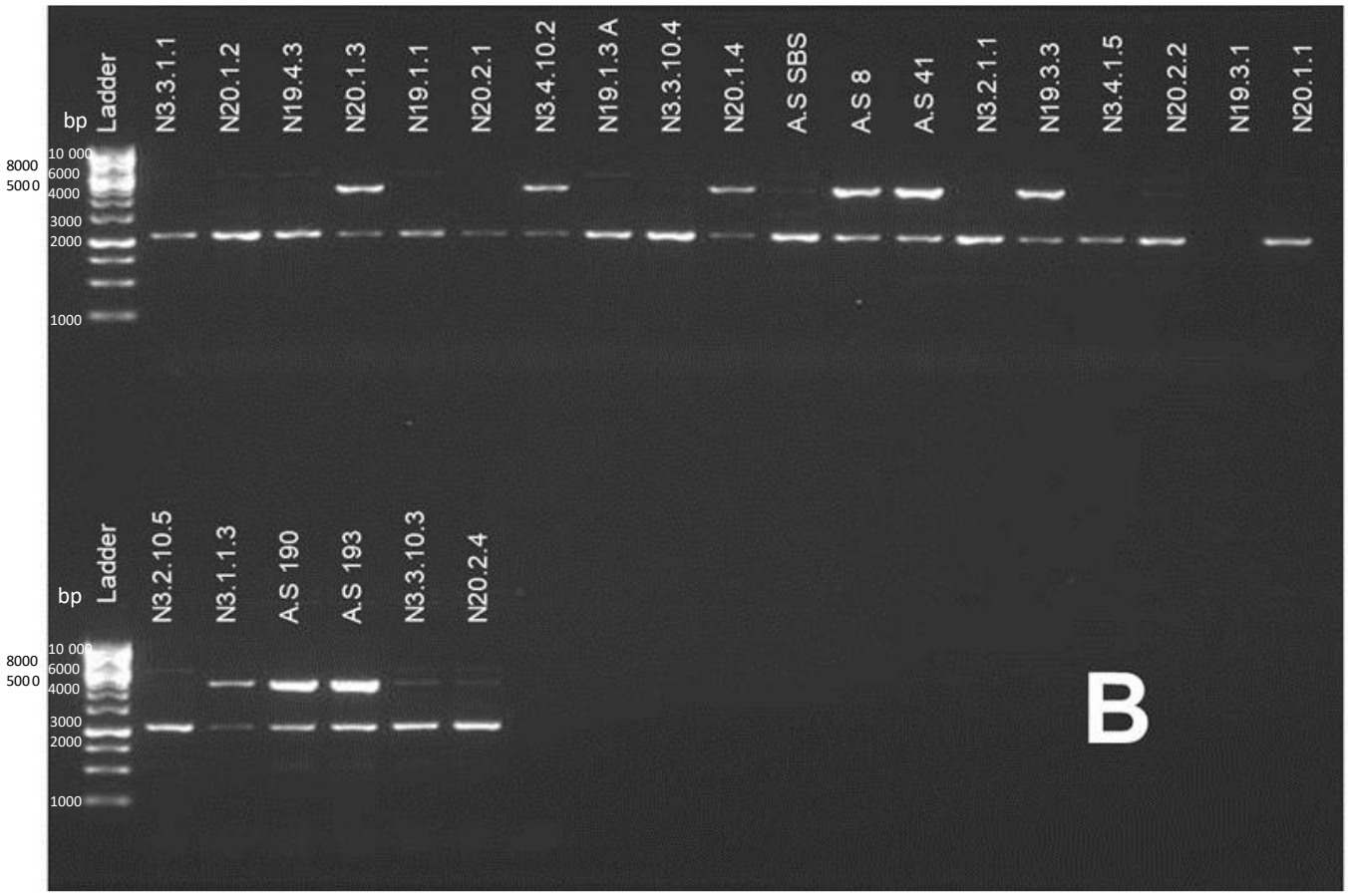


Figure 4: Pictures of the PCR product after electrophoresis. Picture A is overexposed to show all the bands, even the faintest ones. Picture B is the same gel but at optimal UV exposition.



The second time the DNA was extracted from mycelium grown in liquid medium instead of solid agar plates. However, no mycelial growth was found for samples N20.2.4, N20.1.4 and N19.3.1. No absorbance ratio was above 3.0 (Table 3). However, the concentrations were still low and at times lower than the first extraction.

Table 4: The concentration and absorbance ratios of the DNA samples from the second DNA extraction involving liquid media.

SAMPLE NAME	CONCENTRATION [ng/µl]	260/280	260/230
N3.3.10.4	16.4	1.79	1.38
N3.3.10.3	14.6	1.76	1.06
N3.2.1.1	11.2	1.62	1.30
A.S 193	17.0	1.83	1.54
N19.1.1	18.9	1.74	1.42
A.S 41	11.2	1.72	1.33
N3.4.1.5	16.5	1.83	1.43

N3.3.1.1	18.9	1.82	1.45
A.S SBS	26.9	1.77	1.14
N19.3.3 A	7.5	1.59	1.31
N19.1.3	12.1	1.75	1.26
N19.3.3 B	32.5	1.94	0.92
N20.2.1	9.7	2.32	0.65
N20.1.2	10.6	1.83	1.38
N20.1.3	23.4	1.88	0.71
A.S 190	11.5	1.70	1.25
N20.2.2	10.9	1.55	1.14
A.S 8	18.9	2.11	0.87
N3.2.10.5	8.3	1.70	1.24
N3.4.10.2	7.4	1.93	1.13
N19.4.3	7.6	1.55	1.20
N3.1.1.3	13.2	1.57	1.36

PCR was conducted with the chosen (i.e. better quality) replicate for each sample. 30 ng/µl of DNA was used per reaction where possible. For some of the samples it was not possible to reach the 30 ng/µl due to low DNA concentration, and for those as much as possible were used to fulfill the requirements, however, not more than the volume restrictions. PCR products were run on a 2% agarose gel. There were no DNA bands observed for any of the samples, even though there is a trace of DNA at the bottom of the gel (the faint white smudge), indicating the PCR reaction was not successful. The PCR was repeated twice with the same outcome of no bands on the product of electrophoresis.

The PCR was repeated once more using the same protocol. We discovered that the problem with the first two reactions was too little water in the reaction mix. I forgot to add water to each tube to make the reaction volume up to 25 µl. After the water volume was corrected the band corresponding to the *SdhB* gene (1.1kb) was present in all samples except samples A.S. 41 and N19.3.1. Those two samples were, therefore, removed from further investigations.
The PCR products were re-run on a 2% gel with large wells allowing to load larger volumes of the sample and excised from the gel with a scalpel under UV light. The excised bands were purified and analysed on the NanoDrop.

Table 5: The concentration and absorbance ratios of the DNA samples after gel purification.

SAMPLE NAME	CONCENTRATION [ng/µl]	260/280	260/230
N3.3.1.1	5.2	1.69	0.02
N20.1.2	7.9	3.13	0.03
N19.4.3	4.5	2.02	0.03
N20.1.3	5.6	1.98	0.33
N19.1.1	4.2	2.99	0.02
N20.2.1	4.2	2.06	0.67
N3.4.10.2	4.8	1.89	0.34
N19.1.3 A	7.7	1.69	0.57
N3.3.10.4	6.6	1.80	0.81
N20.1.4	3.5	2.15	0.54
A.S SBS	7.6	1.73	0.14
A.S 8	4.5	1.84	0.13
N3.2.1.1	4.9	1.52	0.17
N19.3.3 A	6.1	1.85	0.23
N19.3.3 B	5.5	1.77	0.10
N3.4.1.5	5.7	1.91	0.08
N20.2.2	4.1	1.70	0.13
N20.1.1	9.6	3.35	0.01
N3.2.10.5	5.3	2.16	0.79
N3.1.1.3	4.5	2.21	0.22
A.S 190	6.6	2.17	0.55
A.S 193	4.4	2.51	0.92
N3.3.10.3	8.4	2.09	0.11
N20.2.4	5.5	1.91	0.16

Since both the concentrations as well as the purity of the samples were not satisfactory, we decided to run the purified excised bands on a 2% agarose gel. Even though the B gene band is the main band (very bright, thick and well-defined) in each sample, there were still some other bands present in all of them, even after cutting out the gel bands (Figure 4).

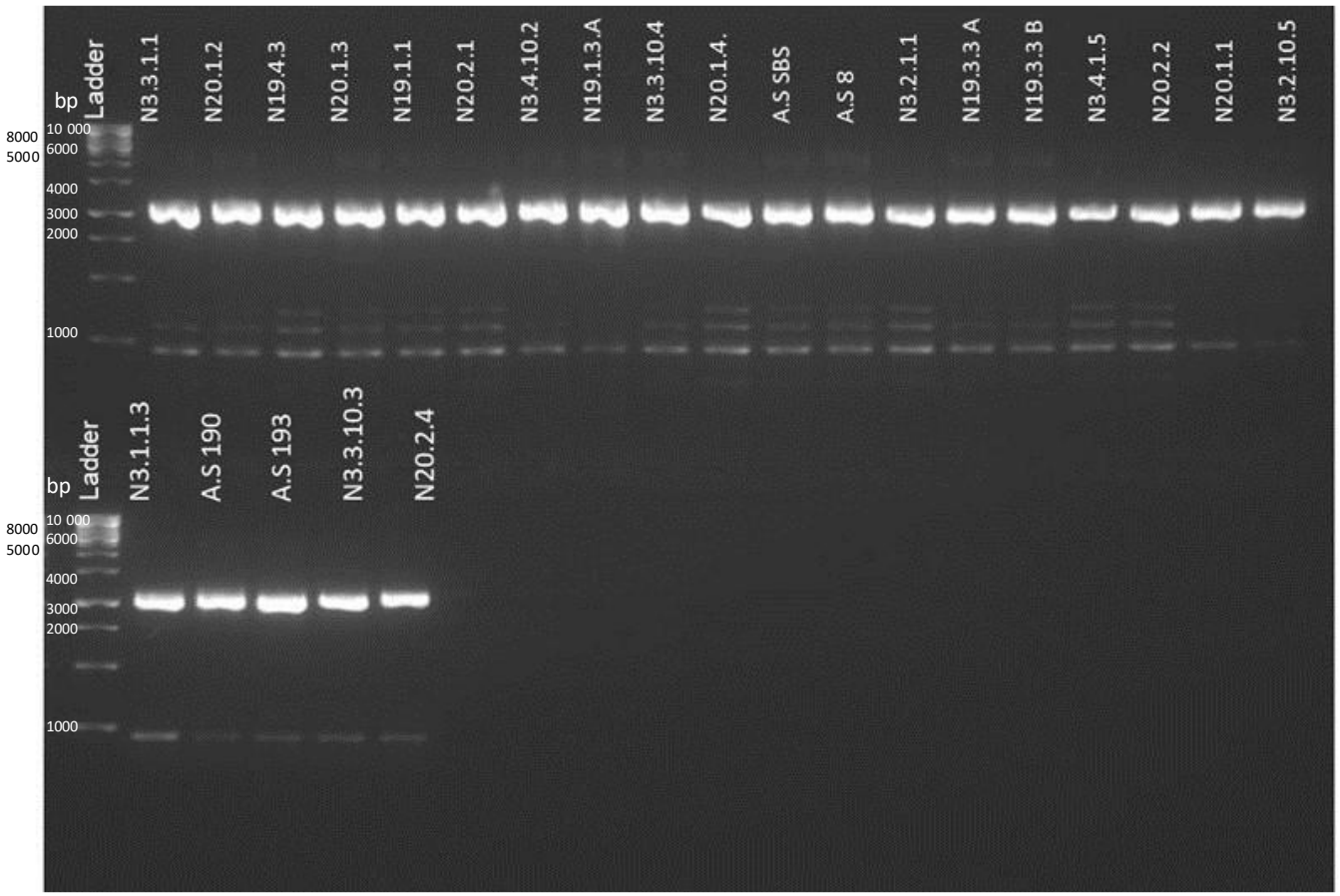


Figure 5: The B-genes on a 2% agarose gel.

The purified *SdhB* gene samples were then used as a template in yet another PCR reaction. Since the DNA concentrations of those template samples were so low (Table 4) each reaction was run in three tubes instead of one and then mixed together, in order to increase the amount of DNA in the final samples. The mixed samples were analyzed on an agarose gel (Figure 5).

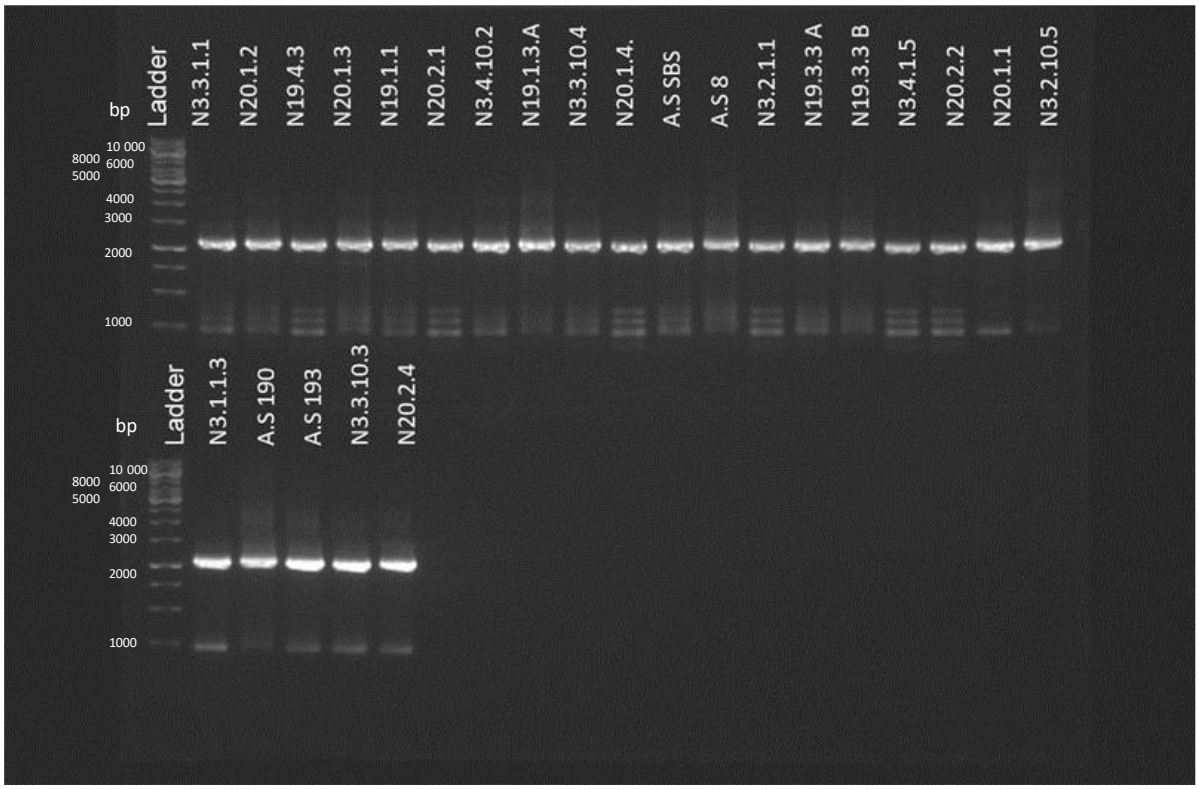


Figure 6: An overexposed picture of the final PCR reaction products.

In Figure 5 the *SdhB* genes are easy to find, however, there are still some unspecific bands seen. Since the other bands seem very difficult to remove and they are very faint in comparison to the target gene, we have decided to use these samples. All samples were precipitated with ethanol and re-dissolved in MQ water, to increase the concentration as well as sample purity. The final concentrations and absorbance ratios of all samples are presented in Table 6.

Table 6: The concentration and absorbance ratios after the PCR product precipitation.

SAMPLE NAME	CONCENTRATION [ng/µl]	260/280	260/230
N3.3.1.1	527.6	1.72	1.62
N20.1.2	508.5	1.78	2.02
N19.4.3	549.4	1.76	1.93
N20.1.3	516.2	1.79	1.98
N19.1.1	525.5	1.77	2.02
N20.2.1	478.5	1.74	1.84
N3.4.10.2	543.4	1.69	1.51
N19.1.3 A	322.6	1.81	2.11

N3.3.10.4	408.9	1.77	1.99
N20.1.4	446.8	1.76	2.10
A.S SBS	473.8	1.78	2.12
A.S 8	503.7	1.70	1.49
N3.2.1.1	432.3	1.75	1.94
N19.3.3 A	476.6	1.71	1.76
N19.3.3 B	468.1	1.78	1.87
N3.4.1.5	372.4	1.76	2.07
N20.2.2	363.1	1.77	2.15
N20.1.1	309.5	1.80	2.03
N3.2.10.5	570.2	1.62	1.27
N3.1.1.3	464.5	1.80	2.11
A.S 190	460.1	1.82	2.10
A.S 193	502.9	1.80	1.92
N3.3.10.3	22.4	1.77	1.86
N20.2.4	457.5	1.81	2.12

In Table 6 the concentration as well as absorbance has increased radically. These samples were then sent away for sequencing at Eurofins Genomics, Germany.

Unfortunately, the sequencing was not successful and no informative data was retrieved from any of the samples. The sequence chromatograms did not produce definitive peaks indicating that the samples contained a mixture of various sequences.

Discussion

Growth

Fluopyram had a noticeable effect on the growth of all the samples which supports the usage of fluopyram and its effectivity against *A.solani* (Lewontin, 1974; Pscheidt & Stevenson, 1988). However, the growth speed varied between the different years and treatment which indicates a lower sensitivity (figure 3). None of the analyzed samples stopped growing completely at 1 µg/ml of fluopyram which is a high dose of fungicides (Dekker, 1995; Avenot

& Michailides, 2010). The samples that were treated showed different growth rate reduction at 1 µg/ml for each coming year, which would indicate less sensitivity against the fluopyram. Furthermore, if the trend that are shown in figure 3 keeps evolving the preparation Propulse SE250 would have less effectivity against the *A.solani* with each year it is used (Mallik et al., 2015). Among the wild type samples the growth rate reduced to 50% at 1 µg/ml of fluopyram which showed the highest sensitivity among the tested samples.

For further investigation more samples should be tested within more different concentrations of fluopyram, to reach a better understanding which concentrations allows the *A.solani* to continue living and adapt more to its environment. A few higher concentrations should also be investigated to understand which level of concentration obliterate the growth completely of the *A.solani*.

DNA analysis

DNA was extracted in two different ways, one from freeze dried mycelia and the other the isolates were grown in liquid media and frozen in liquid nitrogen. Both ways showed the same relatively low DNA concentrations as well as purity, allowing for improvement.

Amplification of the *SdhB* gene proved very tedious; after numerous purifications and precipitations of the samples the gel electrophoresis still showed multiple bands. The reason could be that the primers were not specific to the *SdhB* gene and that they amplified other DNA fragments from *A. solani*. In fact, a BLASTn search against the *A.solani* genome yielded many matches with high percent identity and sequence coverage. The primers chosen in this project were used for sequencing of the *SdhB* gene before (Mallik et al., 2015) and thus we decided to use those primers rather than designing our own. The lack of specificity of the primers could perhaps be overcome by more thorough purification of the desired fragment. Another alternative would be to use nested PCR. If the primers had been designed for this purpose only the results could have shown a different result due to those that were used were not specific enough. The nested PCR would be a good alternative because its purpose is to reduce the non-specific binding (Van Pelt-Verkuil et al., 2008).

Due to unspecific amplification in the final DNA samples, the resulting sequences were not good enough to analyze and thus no conclusions could be drawn as to whether a mutation has occurred and if it is the same as for boscalid resistant isolates. Some studies have been done on in-vitro isolates of *A.solani* (Whartonet al 2012; Metz et al 2019) both times it showed resistance to the other SDHI fungicide boscalid. Metz et al (2019) also concluded a study in the field that showed that when the *A.solani* isolates that showed mutation traits where inoculated the fungicide efficiency where significantly reduced.

Avenot & Michailides (2009) conducted a study in California on *Alternaria alternata* that causes the late blight disease on pistachio. The *A. alternata* isolates that were used showed resistance to boscalid but sensitivity to fluopyram. This shows that even if fluopyram and boscalid belong to the SDHIs they are not the same preparation. This could mean that the use

of fluopyram on these boscalid resistant varieties can be a solution, however, it should always be used with caution, so fungal pathogens keep from developing resistance to fluopyram in the future. For further investigation some studies should be done on those *A.solani* cultures that shows resistance or sensitivity to the high dosages of the fluopyram to try those on different concentrations of the boscalid. This would show if there is any correlation between resistance to one of the two preparations and if that automatically lead to the same behavior to both of the preparations.

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Appendix 1

Sample 1	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	11	15	20	24	28	31	34	37	40	42	47	52	55	3,85
0,02 µg/ml	7	10	13	17	21	23	26	28	31	34	37	41	44	46	3,11
0,06 µg/ml	8	10	13	18	22	25	30	35	38	41	45	50	55	59	4,05
0,12 µg/ml	6	8	9	12	15	16	20	24	28	32	35	40	45	46	3,26
1,0 µg/ml	6	6	7	9	11	15	17	19	21	23	25	28	30	32	2,20

Sample 2	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6mm	9	16	22	27	34	41	48	52	58	62	66	70	73	5,61
0,02 µg/ml	7	11	15	20	24	28	32	36	39	44	47	51	55	61	4,12
0,06 µg/ml	6	10	13	18	23	29	35	41	45	51	55	61	66	72	5,15
0,12 µg/ml	6	8	10	14	17	21	26	30	35	41	45	49	52	54	4,02
1,0 µg/ml	6	6	7	9	11	14	16	18	20	24	26	30	33	36	2,42

Sample 3	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6mm	10	14	20	25	32	37	41	45	51	55	60	65	72	5,22
0,02 µg/ml	6	10	14	18	21	28	33	38	43	49	55	59	62	70	4,96
0,06 µg/ml	5	9	14	20	25	31	36	41	45	51	55	60	65	71	5,08
0,12 µg/ml	6	9	13	18	23	30	35	39	44	49	55	59	63	68	4,94
1,0 µg/ml	6	8	11	16	21	27	32	36	42	48	55	57	59	64	4,80

Sample 4	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6mm	11	15	21	26	32	37	42	47	52	57	62	67	70	5,23
0,02 µg/ml	6	11	15	20	25	30	35	40	43	47	51	54	57	62	4,30
0,06 µg/ml	6	11	15	21	26	35	38	41	47	53	59	61	62	67	4,85
0,12 µg/ml	5	10	14	19	24	30	35	40	43	47	51	55	58	61	4,41
1,0 µg/ml	5	9	13	17	21	26	29	31	34	37	40	44	47	50	3,40

Sample 5	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6mm	11	15	21	26	32	39	45	49	53	57	63	69	73	5,41
0,02 µg/ml	6	10	14	19	24	30	34	37	41	45	50	54	58	62	4,34
0,06 µg/ml	6	19	14	19	24	30	36	42	47	52	58	63	67	72	5,05
0,12 µg/ml	6	9	12	16	20	26	31	35	40	46	50	54	58	65	4,61
1,0 µg/ml	6	8	9	13	16	19	21	23	25	28	31	33	35	39	2,52

Sample 6	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6mm	10	14	20	25	32	39	45	49	54	59	65	70	75	5,65
0,02 µg/ml	5	9	13	18	23	28	33	38	42	47	52	58	63	68	4,89
0,06 µg/ml	5	9	12	16	20	26	31	36	40	45	50	55	60	67	4,76
0,12 µg/ml	5	7	9	14	19	19	23	27	31	35	40	45	49	55	3,82
1,0 µg/ml	5	5	6	18	9	10	11	12	14	16	18	20	21	25	1,34

Sample 7	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	5 mm	10	15	20	25	32	38	43	47	51	56	61	65	69	5,16
0,02 µg/ml	6	11	15	20	25	31	36	41	45	49	54	57	60	67	4,65
0,06 µg/ml	6	11	15	20	25	31	38	44	48	52	57	62	66	70	5,08
0,12 µg/ml	5	9	13	18	23	29	34	39	42	46	50	55	60	64	4,59
1,0 µg/ml	5	9	13	17	20	25	32	39	41	44	47	52	56	60	4,33

Sample 8	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	11	16	21	25	31	36	40	44	49	54	60	65	70	5,04
0,02 µg/ml	6	10	15	20	24	27	29	32	35	38	42	47	52	57	3,66
0,06 µg/ml	5	10	15	19	23	28	31	33	37	41	45	49	53	59	3,92
0,12 µg/ml	5	9	13	17	21	25	28	31	34	38	42	46	50	52	3,62

1,0 µg/ml	5	8	11	15	18	20	23	25	28	31	35	38	40	46	2,99

Sample 9	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	7 mm	12	16	22	27	32	37	41	45	50	55	60	65	70	5,01
0,02 µg/ml	6	10	14	21	27	27	30	32	34	37	40	45	50	56	3,46
0,06 µg/ml	6	9	13	21	28	28	33	37	41	45	49	55	60	67	4,52
0,12 µg/ml	6	9	11	14	17	20	22	24	27	31	35	40	45	49	3,20
1,0 µg/ml	5	6	7	9	11	14	16	17	19	21	23	26	28	34	2,10

Sample 10	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	10	14	20	26	32	38	44	47	51	55	60	65	70	5,16
0,02 µg/ml	6	11	15	20	24	29	34	39	43	47	52	47	62	67	4,46
0,06 µg/ml	5	10	14	19	23	29	34	38	42	47	52	57	62	67	4,75
0,12 µg/ml	5	9	12	17	22	24	28	31	35	39	44	48	51	54	3,81
1,0 µg/ml	6	8	9	15	20	22	25	27	31	35	39	42	45	48	3,35

Sample 11	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	9	12	17	22	25	28	30	33	37	40	46	51	55	3,84
0,02 µg/ml	6	8	10	14	18	21	23	25	28	31	35	39	43	48	3,14
0,06 µg/ml	6	8	10	15	20	25	28	31	35	39	43	48	53	60	4,09
0,12 µg/ml	6	8	10	14	17	20	23	25	28	31	35	39	42	48	3,13
1,0 µg/ml	6	8	9	12	15	19	22	25	28	31	35	39	42	47	3,18

Sample 12	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	10	14	19	23	25	29	32	36	40	44	48	51	58	3,97
0,02 µg/ml	6	8	10	16	22	25	28	30	34	38	43	48	52	57	3,93
0,06 µg/ml	7	12	16	21	25	31	35	38	43	48	53	57	61	67	4,54
0,12 µg/ml	6	10	13	17	21	24	24	23	28	34	40	44	47	52	3,34
1,0 µg/ml	6	8	9	13	16	20	23	25	28	31	35	38	41	45	3,05

Sample 13	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	11	15	20	25	30	33	35	38	41	45	51	56	60	4,13
0,02 µg/ml	6	11	15	19	22	25	28	30	33	36	40	45	50	51	3,36
0,06 µg/ml	6	11	15	20	25	30	35	40	43	46	50	55	59	65	4,43

0,12 µg/ml	6	10	14	17	20	22	25	27	30	34	38	42	45	46	3,08
1,0 µg/ml	6	8	10	13	16	20	23	25	27	30	33	37	40	43	2,88

Sample 14	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	10 mm	12	14	18	21	27	30	33	36	39	42	47	52	58	3,91
0,02 µg/ml	10	13	15	18	21	24	28	32	34	37	40	43	45	60	3,35
0,06 µg/ml	10	10	11	18	24	25	29	32	36	40	45	50	55	63	4,04
0,12 µg/ml	10	12	13	17	20	21	24	26	29	33	37	42	46	51	3,07
1,0 µg/ml	11	12	12	14	15	18	22	25	25	32	36	41	45	45	2,92

Sample 15	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	11	15	20	25	31	36	40	44	48	53	58	62	67	4,85
0,02 µg/ml	6	11	15	20	25	30	35	39	43	47	52	56	60	65	4,51
0,06 µg/ml	6	11	15	21	26	33	38	42	47	52	58	64	70	75	5,32
0,12 µg/ml	6	11	15	20	24	32	36	39	44	49	54	57	59	64	4,53
1,0 µg/ml	6	10	13	17	20	27	31	34	39	44	50	53	55	65	4,42

Sample 16	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	11	16	21	25	30	34	38	42	47	52	57	62	68	4,79
0,02 µg/ml	6	11	15	20	25	30	34	38	42	46	50	55	59	64	4,39
0,06 µg/ml	6	11	15	21	26	32	37	42	46	51	56	62	68	70	5,05
0,12 µg/ml	6	10	14	19	23	27	31	35	39	43	47	51	55	60	4,10
1,0 µg/ml	6	8	10	14	17	21	23	25	28	31	35	39	43	47	3,12

Sample 17	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-Value
0 µg/ml	7 mm	11	15	20	25	31	36	41	45	50	55	61	67	72	5,24
0,02 µg/ml	7	11	15	20	25	30	35	40	44	48	53	59	65	70	4,84
0,06 µg/ml	6	11	16	22	27	34	39	44	49	54	60	66	72	76	5,45
0,12 µg/ml	7	11	15	20	25	30	34	38	42	46	50	55	60	65	4,41
1,0 µg/ml	5	8	10	15	19	25	30	34	36	38	40	47	54	57	4,00

Sample 18	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	6 mm	10	14	18	21	26	30	34	37	40	44	48	52	57	4,01
0,02 µg/ml	7	11	15	19	23	24	27	30	33	36	40	44	48	50	3,23

0,06 µg/ml	7	11	15	21	26	33	38	42	46	51	56	61	65	73	5,01
0,12 µg/ml	6	11	15	18	21	25	27	29	31	34	37	41	44	50	3,05
1,0 µg/ml	6	10	13	17	20	21	23	25	27	30	33	37	40	45	2,71

Sample 19	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	7 mm	8	8	15	21	26	31	35	39	43	47	52	57	60	4,60
0,02 µg/ml	6	10	13	17	20	25	28	31	34	38	42	46	50	58	3,75
0,06 µg/ml	6	11	15	19	23	27	31	35	39	43	47	53	58	67	4,37
0,12 µg/ml	7	12	17	22	27	33	38	42	46	50	55	61	67	74	4,97
1,0 µg/ml	6	9	11	15	19	22	24	26	29	32	35	39	42	45	2,97

Sample 20	Day														
Conc.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	K-value
0 µg/ml	8 mm	13	17	22	27	32	36	39	43	48	53	57	60	62	4,49
0,02 µg/ml	8	12	16	19	21	32	37	42	47	52	57	61	65	70	4,98
0,06 µg/ml	6	8	9	17	24	31	36	40	44	49	54	59	64	69	5,08
0,12 µg/ml	5	7	8	13	18	25	26	27	27	29	31	38	45	48	3,15
1,0 µg/ml	5	6	6	8	10	16	18	20	22	24	27	31	35	36	2,57

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