Investigating fungicide resistance of *Alternaria solani*, a *Solanaceae* pathogen, on potato crops in south-eastern Sweden and its relationship with soil

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Investigating fungicide resistance of *Alternaria solani*, a *Solanaceae* pathogen, on potato crops in south-eastern Sweden and its relationship with soil

Etude de la résistance aux fongicides d'Alternaria solani, un pathogène des Solanaceae, sur les cultures de pomme de terre dans le sud-est de la Suède ainsi que sa relation avec le sol.

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Abstract

Alternaria solani is a worldwide present fungus that can cause a lot of damage such as early blight in potato crops. Potato can suffer a lot of yield losses because of this pathogen, and the main method used today to fight against it, is the application of fungicides. Yet fungicides are chemicals that contaminate the environment and are not assimilated so they can be found in water for example, which can cause problems for human health as well. As the climate is becoming warmer and warmer, it is also expected that the *A. solani* severity will increase, which will induce more use of fungicides. *A. solani* resistance to fungicides has been reported and seems to develop quickly. Firstly, it is important to study *A. solani* resistance development to new fungicides as it may improve the possibility for early detection of fungicide resistance. In this study, we looked at the mycelial growth of *A. solani* field isolates by increasing the concentration of fluopyram, a fungicide, in two growth assays (liquid and solid). Secondly, to be able to predict the occurrence of early blight it is also important to investigate the survival and growth of *A. solani* in the soil. Here we did a soil assay where we incubated *A. solani* infected rye kernels in different soils under differents conditions (unsterilized wet, unsterilized dry or sterilized) followed by a qPCR performed on the rye kernels to assess the *A. solani* quantity. In our experiment, the growth of *A. solani* decreased with the increase of fluopyram concentration. Moreover, the growth reduction with decreased fluopyram concentration was reduced for the later collection year, which indicates that the sensitivity to flupyram decreased over the years. The growth of *A. solani* in the soil was affected by the said soil condition (dry, sterilized or unsterilized). Our results are pointing to *A. solani* surviving better in dry soils which is concerning knowing the expected temperature rise, as well as the drought experienced today but also in the future.

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Abbreviations

UN	United Nations
FAO	Food and Agriculture Organisation of the United Nations
SCB	Statistikmyndigheten (Statistic Sweden)
A. solani	*Alternaria solani*
IPCC	Intergovernmental Panel on Climate Change
IEA	International Energy Agency
SDHI	Succinate Dehydrogenase Inhibitor
IPM	Integrated Pest Management

Introduction

1.1 World hunger

The world is changing and evolving toward a place where more food will be needed years after years, however the access to this resource can be complicated and unfair. The demand for food and accessibility to this resource is already a problem and as the world population is expected to increase by 1.7 billion in 2050 (UN a) the need will only expand in order to sustain the society.
In 2023 food insecurity affects 345 million people and has increased rapidly in the last years (World food programme, 2023). World hunger affected between 702 and 828 million of people worldwide in 2021 (FAO, 2022).

1.2 UN objectives and importance of potato crops

The world hunger question is addressed and solutions have been proposed. Indeed, the second of the UN sustainability goals, that have been developed by United Nations Conference on Sustainable Development in 2012 , is “Zero hunger” (UN b). The objective is second among the 17 other objectives. Objective 1, which is “No poverty”, is intrinsically linked to the second one (UN b). One of the major elements to meet these objectives could be potatoes. Potatoes are a source of high quality food, as they provide macronutrients (amylopectin and amylose), vitamins (C and B), potassium, or magnesium (Beals, 2019), which are much needed for a balanced human diet. It is also the crop that produces more yield per field unit than any other crop (FAOStat, 2022). Thus, it seems that potato is very suitable to fight world hunger and poverty. Potato is also used for starch production, 2.5 million tons of potato starch are produced worldwide according to Semeijn and Buwalda (2018). Starch is used in the food industry as well as for paper production and in the paint industry. In Sweden, table potato crops represent 74% of all potatoes cultivable area (Eriksson et al., 2016). The total potato yield in Sweden in 2022, were up to 74 980 kg per hectare (SCB, 2022).

1.3 Potato diseases

Potatoes are subject to a lot of different types of diseases, and these diseases have been reported all around the world. In order to use potatoes to fight the world hunger and poverty it is needed to protect this crop. Plant diseases cause up to 15% of crop losses, and up to 80% of those are due to pathogenic fungi (Peng et al., 2021).
Some of the most important diseases are late blight caused by *Phytophthora infestans*, wart caused by *Synchytrium endobioticum*, or early blight that can be caused by *Alternaria* species such as *Alternaria solani* (Adolf et al., 2020), but also diseases caused by bacteria such as blackleg caused by *Dickeya* (Charkowski et al., 2020). Potatoes are also infected by viruses like potato virus Y, a Potyvirus, which is transmitted by aphids or the potato aucuba mosaic virus (Kreuze et al., 2020). Potato viruses can be transmitted by insects, and such insect can also be pests, e.g. aphids or *Liriomyza huidobrensis* (Kroschel et al., 2020).
Alternaria solani fungus can infect plant debris (Kemmitt, 2002) and easily be transferred from crops to other close potential host plants through its spore dispersion (Jindo et al., 2021). The pathogen is not dangerous for humans, nevertheless, *A. solani* can cause a lot of damages to crops as it can induce large yield losses. *A. solani* can be recognized by its lesions that are dark-brownish bullseyes pattern (rings alternation) spots. These lesions can be observed mainly on mature leaves (Van der Walls et al., 2001). Early blight has spread all around Europe, including Sweden, which means a lot of potential infection by the fungus. In southern Sweden, the occurrence of early blight has been studied, and 44% of the potato lesions studied were *A. solani* (Edin et al., 2019).

The soil nutrient content and soil moisture can also influence pathogens, but the exact effect may depend on the pathogen itself (Dordas, 2009). For example, an increase in nitrogen for pear host seems to increase *Erwinia amylovora* development, but a decrease for tomato host seems to increase *A. solani* development (Ghorbani et al., 2008). The relationship between potassium soil content and disease severity is even more complicated, with low levels of potassium increasing the severity of various diseaseas, including early blight, while high potassium content has the opposite effect (Dordas, 2009).
Moreover, soil moisture can be linked to severity and abundance of diseases (Ghorbani et al., 2008). Interestingly, a recent study on early blight in potato farms in south Sweden showed a strong positive association between infection rate and a high sand content (Stridh et al. unpublished data).

1.4 Climate change affects crop cultivation practices

In addition to the previously addressed problems, climate change has an important part in the potato cultivation. The temperature has continuously increased, with an

increase of surface temperature of 1.1 °C from 1850-1900 to 2011-2020 (IPPC, 2023). The temperature increase is expected to reach 1.5°C in the near future (IPPC, 2023). The CO_2 emissions are also increasing and since 1900 the CO_2 emission increased up to 36.8 GT in 2022 for energy combustion and industrial processes (IEA, 2023). Compared to other crops, potatoes are relatively environmentally friendly. In 2020 it was shown that potato crops emitted 13.7175 kt of N_2O compared to rice that emitted 173.1862 kt or wheat that emitted 188.078 kt (FAOStat, 2022). Climate change is intricately linked to pathogen development and spreading. The increasing CO_2 levels as well as the increase in temperature will probably influence plant pathogens, as seen with *Alternaria spp.* infections on cultivated rocket (Gullino et al., 2018). Both factors were shown to lead to an increase of the disease occurrence (Gullino et al., 2018). The temperature rise will also likely induce more fungal pathogens existence in soil (Delgado-Baquerizo et al., 2020), which could lead to an increase of plant infection. *Alternaria solani* survival also depends on the moisture and temperature in the soil, as it can survive from -3.3 °C to 21.2 °C (Jindo et al., 2021). Increased occurrence of the pathogen was recorded in the last years.
Thus, a need for more intense culturing practices, required to increase the yield, will lead to more chemical products such as fungicide or pesticide being used.

1.5 Fungicides are a problem for environment and human health

Potato production relies mainly on the use of fungicides to combat diseases. In fact potato crops are among the most treated ones in the world (Yuen, 2021). The use of fungicides on crops is a method used for decades, but has shown its limitations today. While the use of fungicides is an easy way of getting rid of a lot of pathogens and pests, the treatments are mainly chemical and lead to many environmental problems. Fungicides are harmful to soils by affecting the present organisms, but also their diversity and enzymes (Baćmaga et al., 2016). Fungicide presence can be detected in soil water as well as surface water (Reilly et al., 2012). It indicates that fungicides are not completely degraded in water, and they can be harmful for aquatic organisms (Gustafsson et al., 2010, Zubrod et al., 2019).
It has been reported that the use of fungicides affects also human health. They were linked to diseases such as cancer, asthma, diabetes, leukemia or neurocognitive diseases, as described in detailed in Kim *et al.* (2017).

1.6 Integrated Pest Management as an alternative to pesticides

One possible solution to reduce the extensive fungicide use is to apply integrated pest management methods (IPM). IPM methods reduce the utilization of pesticides and favour a more natural pest control (European Commission a). IPM aims to control the diseases with a combination of all known methods (Stenberg, 2017). It promotes a more reasonable use of fungicides and pesticides, the use must be motivated, and the ecological, economical and human health aspects must be considered (European commission a).
The adoption of the IPM methods is mandatory within EU and it is expected by the European Commission to reduce pesticide use by 50% by 2030 (European Commission b). Moreover, the use of these methods offers interesting perspectives and favourable results.
IPM are already employed in potato cultivation in many countries. The use of biological control agents, such as Carabid beetles to fight against some pest, and adopting IPM strategies were shown to increase farmers' safety, to lower the rate of fungicide resistance development and to lower food contamination (Horne & Page, 2022). However, IPM methods are still not used frequently and wildly enough (Horne & Page, 2022).

1.7 Alternaria solani

1.7.1 Species Description

Among all plant pathogenic fungi, the *Alternaria* genus is a really important one, as it contains a lot of species that cause economically important plant diseases. This genus includes *A. solani* that causes early blight, but also *A. alternata* that causes black spot (Troncoso-Rojas & Tiznado-Hernández, 2014). *Alternaria solani* is a soil-borne fungus that can be found all around the world. It can infect crops such as tomatoes (Chaerani & Voorrips, 2006), eggplants (Darwish et al., 2014), potatoes (Abuley et al., 2018) or other crops from the *Solanaceae* family. The lesions induced by *A. solani* can be recognised by the alternating black rings also referred to as "bullseyes" (figure 1) (Kemmitt, 2002). *Alternaria solani* spores (figure 2 and 3) are asexual and can germinate when the right conditions are met. Conidia can be dispersed by wind or water (Kemmitt, 2002) and are easily conserved over seasons, mostly in dry soil (Jindo et al., 2021). The spores can germinate on a leaf when there is humidity and the right temperature is met (Kemmitt, 2002).

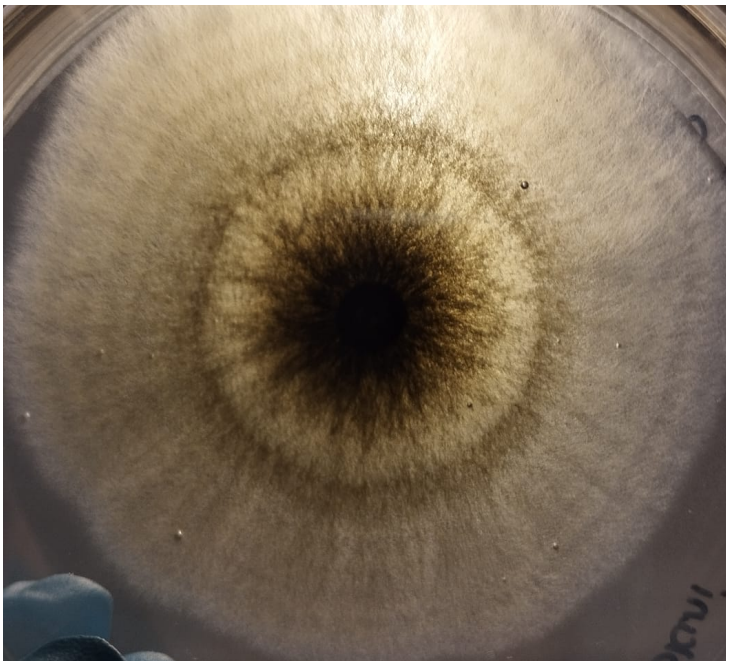


Figure 1. Alternaria solani solid medium culture with the typical growth pattern

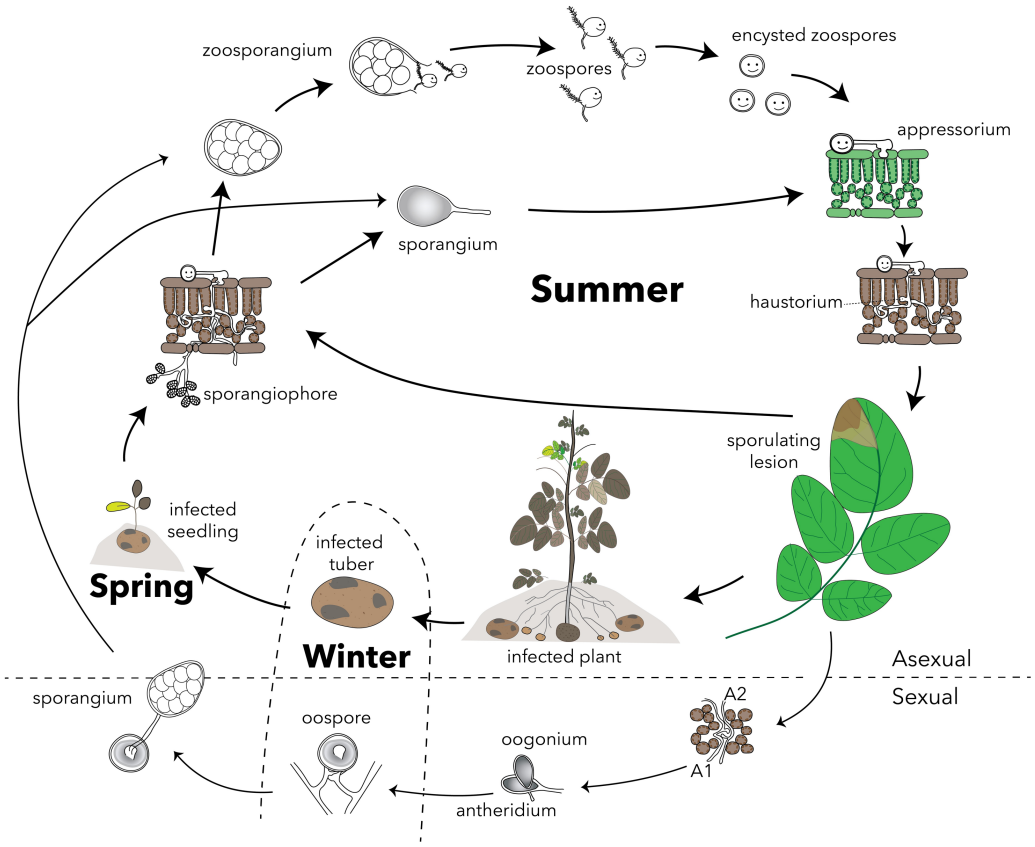


Figure 2. Lifecycle of Alternaria solani (Brouwer, 2021).

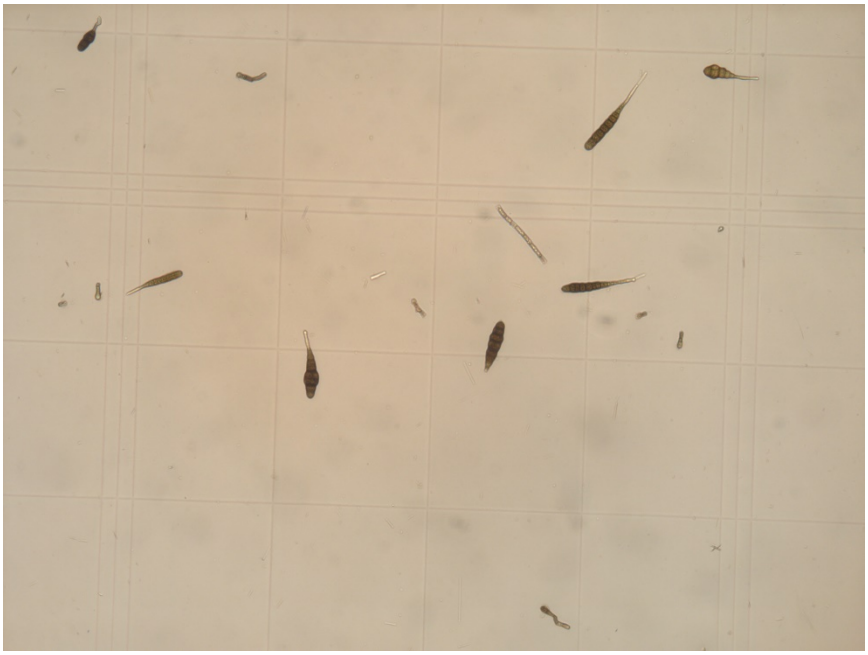


Figure 3. Alternaria solani spores under light microscope, 200 x magnification

1.7.2 *A. solani* mutation and resistance emergence

One way to treat early blight is to use fungicides, such as SDHI or QoI. SDHI fungicides target the succinate dehydrogenase enzyme (Avenot & Michailides, 2010) and QoI fungicides are quinone outside inhibitors, both very effective against early blight. However due to the intense use of both types of fungicides, resistance developed in *A. solani* strains in the fields (Gudmestad et al., 2013; Mostafanezhad et al., 2022). Such fungicide resistance occurs mainly due to mutations. Different mutations have been studied in *A. solani*, such as the sdh mutations which are mutations in the Complex II of the respiratory chain (Metz et al., 2019), or F129L mutations of cytochrome b (Leiminger et al., 2014). Mutated strains of *A. solani* are problematic not only because they are resistant to the applied fungicide, but also because they spread fast through clonal reproduction. It means that the resistant strain of *A. solani* can easily be transferred from one crop to another and continue to be a problem for the next culture in the field. The mutations of the *SDH* genes are believed to be involved in resistance development in most strains and their occurrence was reported in *A. solani* samples collected in the EU, including Sweden (Mostafanezhad et al., 2022), and in the USA (Budde-Rodriguez et al., 2021).

One of the most commonly used fungicides against early blight is Propulse, developed by Bayer. It is a new fungicide that has been used for a few years now (first year it was registered was 2020) and constitutes of two components, fluopyram and prothioconazole. Prothioconazole is a triazole that inhibits C-14 demethylation during the ergosterol biosynthesis (Breunig & Chilvers, 2022) and

fluopyram inhibits succinate dehydrogenase**.** Fluopyram inhibits respiration by its binding instead of the ubiquinone on the ubiquinone binding site and thereby blocking it (Avenot & Michailides, 2010). This site is formed by the B, C and D SDH subunits (Rathod et al., 2022). Fluopyram is a SDHI fungicide which is used for plants but also for nematodes (Ji et al., 2019), and is used all around the world (Rathod et al., 2022). This compound is toxic for aquatic life, and while Propulse is mainly applied through spraying (Bayer Crop Science a), there is a possibility of both plant and soil absorption of the product. *A. solani* is less sensitive to bosclaid than it is to fluopyram (Budde-Rodriguez et al., 2021), however Propulse being a rather new fungicide, *A. solani* resistance to this fungicide is not yet documented.

Understanding how resistance to the newly introduced fungicide Propulse develops in *A. solani* may help us to prevent resistance development to other fungicides in the future. Moreover, it is also important to study every aspect of the interactions between the fungicide, *A. solani*, the host plant and the soil in order to better understand and prevent environmental and health risks.

1.8 Aims

The first aim of this project was to study potential resistance development in *A. solani* to the new fungicide Propulse. Because fungicide resistance to SDHI fungicides is well recorded in *A. solani*, the study focused on fluopyram, one of the active ingredients of Propulse. By using *A. solani* isolates collected from potato field trials from 2018-2022 where Propulse was sprayed, it was possible to look for early signs of resistance development over the years. This may give us a better understanding of early detection of resistance development in *A. solani*, which may help to detect this development and prevent it in the future.
A second aim was to study the survival of *A. solani* in the soil, to help understand how this fungus acts in soil of different types and in different conditions, and what can influence its survival so disease can be prevented in the future.

1.9 Research questions

- Do *A. solani* isolates collected from potato treated with Propulse differ from isolates collected from control treatments in the field trails regarding tolerance to fluopyram, and is there increased tolerance over the years?
- Do *A. solani* isolates develop mutations that induce fluopyram resistance ?
- Does the soil type (higher vs. lower sand content) influence *A. solani* growth and survival?

- Does the soil condition (i.e sterilized or not, watered or not) have an influence on *A. solani* growth and survival?

To answer these questions the experiments were divided into two parts. The first one was growth assays designed to investigate how the *A. solani* isolates collected in different years and under different spraying regimes can grow and sporulate across fluopyram concentrations. The second was soil assay designed to assess the soil type and condition impact on *A. solani* survival.

Materials and Methods

2.1 *Alternaria solani* cultivation

All solid *A. solani* cultures were gown on 20% potato dextrose agar (PDA) medium, supplemented with 25 µg/ml chlortetracycline (CTC), unless specified differently. The liquid cultures were grown in 20% potato dextrose broth (PDB), supplemented with 25 µg/ml CTC.

For the 20% PDA medium, 7.8g of PDA and 12g of bacto agar were mixed and filled up with MilliQ water to obtain for a total of 1 L solution. For the PDB medium, 24g of PDB was added to MilliQ water. The media were autoclaved for 20 min at 121°C.

A stock solution of CTC was made by dissolving 1 g of CTC in DMSO and MilliQ water (1:1, v/v) in order to obtain 100 mg/ml solutions. The stock solution was then aliquoted into 1ml lots and added to the growth media to a final concentration of 25 µg/ml.

2.1.1 Leaf sample isolation

Potato leaves with lesions visually identified as *A. solani*, were collected in different field trials in south Sweden in years 2018 to 2022. The samples went through several re-culturing steps in order to obtain single cell cultures. Briefly, a piece of a lesion from an infected leaf was placed on a water agar plate and incubated at 18 °C and 30% near-UV light from 8 am to 5 pm. The spores produced by the fungi were then collected with a plastic loop and transferred to a 20% PDA plate. The newly inoculated plates were incubated under the same conditions as the first plates. After a 10 days incubation, the plates were looked at under a microscope, spores were localized and collected by pipetting 40 µl of MilliQ water back and forth in order to detach the spores from the mycelium. The droplet was then spread on a new 20% PDA plate and incubated for 3 hours at room temperature in order to

induce germination. A single germinated spore was selected from each culture and transferred, by cutting a piece of the media underneath, to a new plate. The final cultures were single spore cultures, thus different isolates.

2.2 Growth Assays

From the collection of isolates, thirty were selected across all sampling years, when possible six isolates per year, and both from untreated samples and ones collected in the fields treated with Propulse (in combination with the fungicide Narita) (Tab. 1). All the isolates were re-cultured in order for all of them to be of the same age. From this, only 25 isolates grew and were used in the growth assays (two liquid assays and one solid assay). A piece of the original culture was transferred to a new 20% PDA plate. Two duplicates were made for all samples and were keep for two weeks in the dark. These new cultures were used for the growth assays.

Table 1. A table showing the different A. solani isolates used for the growth assays with their name, year of collection and treatment. 100% or 50% Narita/Propulse treatment designates the proportion of fungicide applied on the field.

SAMPLES	YEAR	TREATMENT	FIELD LOCALISATON
N3.3.10.1	2018	Narita and Propulse	Nymö
N3.1.10.1	2018	Narita and Propulse	Nymö
N3.2.10.1	2018	Narita and Propulse	Nymö
N2.4.1.1	2018	Untreated	Nymö
N2.2.1.3	2018	Untreated	Nymö
N2.2.1.4	2018	Untreated	Nymö
N19.1.1	2019	Untreated	Nymö
N19.1.3	2019	Untreated	Nymö
N19.3.3A	2019	100% Narita/Propulse	Nymö
N19.3.3B	2019	100% Narita/Propulse	Nymö
N20.1.3	2020	Untreated	Nymö
N20.2.3	2020	100% Narita/Propulse	Nymö
N21.5.3	2021	Untreated	Nymö
N21.5.1	2021	Untreated	Nymö
N21.7.1	2021	50% Narita/Propulse	Nymö
N21.7.2	2021	50% Narita/Propulse	Nymö
N21.7.3	2021	50% Narita/Propulse	Nymö
N21.5.2	2021	Untreated	Nymö
L22.6.1	2022	100% Narita/Propulse	Lyngby gård
L22.6.2	2022	100% Narita/Propulse	Lyngby gård
L22.6.3	2022	100% Narita/Propulse	Lyngby gård

L22.3.1	2022	Untreated	Lyngby gård
L22.3.2	2022	Untreated	Lyngby gård
L22.3.3	2022	Untreated	Lyngby gård

A stock solution of fluopyram was made by dissolving 4.8 mg of fluopyram in 20 ml of DMSO and MilliQ water (1:1, v/v) in order to obtain a 24 µg/ml solution.

The different concentrations of fluopyram solution used for the growth assays were: 0.00 µg/ml; 0.500 µg/ml; 0.958 µg/ml; 2.66 µg/ml; 4.13 µg/ml; 7.05 µg/ml and 15.0 µg/ml; and will respectively be called: C0, C1, C2, C3, C4, C5, C6. The solutions were made by adding fluopyram stock solution to 20% PDB, supplemented with 25 µl/ml CTC.

2.2.1 Liquid growth assay

For the first liquid assay, the fluopyram solutions (C0 to C6) were aliquoted into multiwell plates and inoculated with the twenty five isolates, so that each isolate was grown at each fluopyram concentration in duplicate, resulting is 350 cultures. The inoculation was performed by cutting a piece of the fungal culture, including the medium, with a 0.4 mm diameter cork borer, to assure equal size of the inoculation plugs (Fig. 4). All work was performed under a sterile hood, in order to keep a sterile environment. The cultures were keep in the dark, at room temperature, until the first isolate in the C0 concentration reached the edges of the wells (between one and two weeks). At this point the growth was recorded by measuring two diameters of the mycelium (left to right and top to bottom) with a ruler.
The four measurements of each culture were used to calculate the mean value and construct a preliminary growth curve for all the isolates. Based on their growth patterns the number of investigated isolates was decreased to fourteen. The fourteen isolates were used in the second liquid growth assay, with the same fluopyram concentrations.

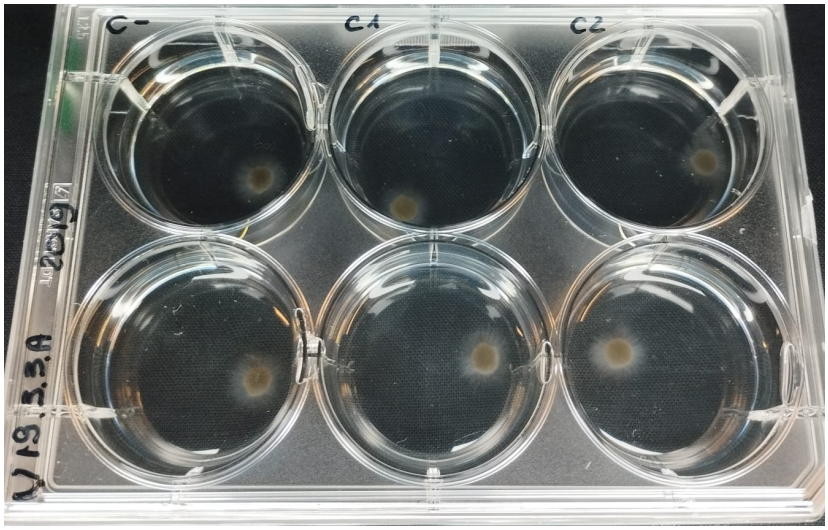


Figure 4. The liquid growth assay setup on a six-well plate. The wells are filled with growth medium supplemented with three different concentrations of fluopyram (C0, C1, C2) and inoculated with small, round plugs of A. solani isolate.

2.2.2 Solid growth assay

The fourteen isolates selected in the liquid growth assays were cultured on 20% PDA plates with the same concentrations of fluopyram as the liquid assay (C0 to C6), two replicates per concentration and per isolate. Pieces of the original cultures were transferred to the new 20% PDA plates and kept in the dark for 6-8 days at room temperature. They were then incubated at 18 °C with 30% near-UV light for 12 days. The growth was measured as before, i.e. two diameters per culture, after 14 days of growth. Moreover, after 12 days it was noted if the cultures were sporulating or not.

2.3 Validation of *A. solani* isolation

2.3.1 DNA extraction

To validate that the collected isolates were *A. solani*, the fourteen isolates selected in the growth assays were grown in 200 ml of 20% PDB medium, amended with 25 µg/ml CTC, in 500 ml bottles. The cultures were incubated in darkness, with mild shaking and at room temperature for 12 days.

The mycelium was then filtered from the medium through a sterile 40 µm mesh and blotted on a filter paper to absorb all the excess medium. The agar plug was removed with forceps and the mycelium was then snap-frozen in liquid nitrogen, directly in a mortar. It was ground with a chilled pestle until a fine powder was

obtained. About 100 mg was transferred to the "disruption tube" provided in the DNeasy Pro Plant Kit (Qiagen, Germany). The remaining powdered mycelium was conserved in an Eppendorf tube in a -80 °C freezer.

The genomic DNA was extracted using the DNeasy Pro Plant Kit (Qiagen, Germany), following the manufacturer's protocol. Briefly, 500 µl of CD1 lysis buffer was added to the powdered mycelium and disrupted with a vortex for five minutes. It was then centrifuged at 10 000 rpm for 2 min. The supernatant was transferred to a new Eppendorf tube and 200 µl of CD2 buffer was added. It was centrifuged again for 1 min at 10 000 rpm. The supernatant was transferred to a new Eppendorf tube, and 500µl APP buffer was added. The solution was vortexed for 5 seconds, transferred to a spin column and centrifuged at 10 000 rpm for 1 min. The flow-through was discarded and 650 µl of AW1 buffer was pipetted onto the column, centrifuged with the same settings as before. The column was placed in a new collection tube, and AW2 buffer was added before centrifugation. The flow-through was discarded again and the column centrifuged a second time for 2 minutes at 15 000 rpm. The column was finally placed in a new Eppendorf tube and the DNA was eluted with 30 µl EB buffer.

After the DNA extraction, the DNA samples were quantified and their quality assessed with the NanoDrop spectrometer. The EB buffer was used as a blank.

2.3.2 PCR

The species identity of the isolated cultures was verified with PCR, following the method described by Kumar *et al.* (2013). Each 1 µl gDNA sample was mixed with 2.5 µl of 10x DreamTaq Green Buffer, 2.5 µl dNTPs, 1µl of each primers, 0.25 µl Dream Taq polymerase (ThermoScientific) and 16.75 µl of nuclease free water. The two primers used were *A. solani*-specific primers AS1 (5'-GCTCCCACTCCTTCCGCGC-3') and AS2 (5'-GGAGGTGGAGTTACCGACAA-3'). The first step of the PCR was denaturation at 95°C for 5 min, next are the 35 cycles of denaturation at 95°C for 1 min, then the annealing step for 1 min at 54°C, and the extension at 72 °C for 1 min. The PCR was finalized with the extension at 72°C for 10 min. (Kumar et al., 2013).

The PCR products were run on a 1% agarose gel, i.e. 1g of agarose was used for 100 ml of TAE buffer. Once it cooled down Gel Red dye was added to the final concentration of 1x, i.e. 5 µL per 50 mL of agarose solution. The gel was then placed in a mould and let dry for 30 minutes. Once dry, the gel was placed in an electrophoresis tank and covered by TAE buffer. The samples were loaded in the gel and the current turned on to allow the samples to migrate.
The gel was placed under UV lights to visualise the migrated sample bands.

2.4 Preparation of soil assays

Soil was collected in November 2022 from fields in four different potato farms in Skåne, Sweden, here denoted HAK (55.8871830°; 14.1439909°), PET (55.5691443°; 14.0421306°), JAR (55.8805570°; 14.1266293°) and VIK (55.580704°; 14.0544581°). Two fields were considered to have clay soil and the two others were considered to have sandy soil. This assumption was confirmed through a soil type and nutrient analysis of the soils done by Eurofins Scientific (Eurofins Agro Testing Sweden AB, Appendix 1). The soils were stored in a cold room for about three months until the start of the experiment.

Soils from the different fields were put in 2L pots in the greenhouse and separated into three groups for each of the four collection sites. The soil in the first and second groups was not altered from its initial condition and is referred to as "unsterilized". Group one was then watered regularly, while soil in group two was left dry for the entire duration of the trial. In the last group to test the effect of reduced competition from other microbes, the soil was sterilized in an oven at 65°C for 6 hours three times, with 20 hours between each time (Löbmann, 2015) . These pots were watered regularly during the experiment. There were seven replicates of each condition and each soil type, resulting in a total of 84 samples.

2.4.1 Rye inoculation of *A. solani* and performance of soil assays

To test growth and survival of *A. solani* in the different soil types and conditions, rye kernels were inoculated with the fungus and dug into the soil for 66 days (from 24th February 2023 to 2nd May 2023) . The infection was conducted as closely as possible to the protocol established by Adolf and Hausladen (2015). 150g of rye kernels were weighed and transferred in autoclavable bags. 80 ml of distilled water was added before the bags were autoclaved twice for 20 min at 121°C. The kernels were then inoculated with a lab strain of *A. solani* (As112) by manually crushing half of a plate of solid culture inside each bag, and incubated at 25°C for two weeks. The inoculated kernels were transferred from the autoclavable bags to nylon bags. The bags were weighed again before being dug into the pots of soil. All the pots were watered, even the ones under the dry condition, for all the soils to start the experiments under the same conditions.

During the running time of the experiment, the soils were watered every 2-3 days (except the ones under the dry condition). Seedlings growing from the soils were kept, as they were considered part of the soil of the collection site.

After 66 days, the bags were removed from the soils. They were weighed and kernels sample of 50ml was taken from each bag that were used for DNA extraction and qPCR. For each treatment (sterilized, unsterilized wet, unsterilized dry) and

each collection site (HAK, PET, JAR, VIK) there were three random samples of kernels taken.

2.4.2 DNA extraction

The DNA extraction and quantification were conducted the same way as for the growth assays samples. To assess if the DNA extracted from the kernels using the Qiagen kit is of sufficient quality it was analysed using NanoDrop spectrometer. Four samples (one for each soil) were also analysed on an electrophoresis gel to check for potential degradation.

2.4.3 DNA clean-up

The quality of some of the extracted DNA samples was not satisfactory, and therefore the samples were purified using the MicroSpin G-50 Columns (Cytiva) following the manufacturer's protocol. A new NanoDrop measurement was performed to assess the purity of the new samples.

2.4.4 Quantitative PCR

The purified DNA samples were used to perform a qPCR, to quantify *A. solani* in the samples. Firstly, the samples were diluted with MilliQ water in order to obtain 50 ng/µl of final DNA concentration for each of our samples.
DNA extracted from As112 lab strain, was diluted in MilliQ water in order to obtain a serial dilution series of 50 ng/µl, 5 ng/µl, 0.5 ng/µl, 50 pg/µl, 5 pg/µl and 0.5 pg/µl. These solutions were used to construct the standard curve for the data analysis.
The qPCR reaction mixture consisted of 10 µl SYBR Green master mix (Applied Biosystems), 0.4 µl 10x F-primer, 0.4 µl 10x R-primer and 7.2 µl MilliQ water and 100ng (i.e. 2 µl) DNA. Each DNA sample was run in triplicate and standards were loaded onto each of the two plates.
The qPCR was performed in BioRad CFX-96 thermocycler following the set up of 95 °C for 10 min followed by 40 cycles of 94 °C for 15s then 54 °C for 10s and 72°C for 20s, next the fluorescence read at 72 °C at the end of each of the 40 cycles, finished by the analysis of the melting curve at 65 – 95 °C (Kumar et al., 2013).

2.5 Data analysis

For data analysis and creation of graphs, the language R was used with R studio as an interface (Version 1.1.456 – © 2009-2018 RStudio, Inc.). Type II sums of squares were used in all analyses of variances (Anovas) and normality of residuals were tested with Shapiro-Wilk normality test. Post-hoc tests were performed to test treatment differences in the Anovas using estimated marginal means with the Tukey's method.

2.5.1 Growth data analysis

2.5.1.1 Mycelium growth

The measurements collected through the growth assays were used to assess the growth of all the isolates according to the concentration of fluopyram, year of collection, and their field treatment (Propulse vs. untreated).

From each measurement, 0.4 cm, i.e. the size of the agar plug, was subtracted in order to obtain only the size of the new culture. The mean of the four measurements for all samples was calculated and standardized by percentage calculation. These results were used to construct the growth curves.

The data from the growth assays was analysed with a linear mixed model Anova with the dependent variable percentage of growth area and involving the fixed factors fluopyram concentration, year of collection, field treatment and their two and three way interactions. The random factor isolate was included to control for repeated measures of the isolates. Because of few isolates included from 2020, this year was excluded from the analysis in the liquid growth assay.

2.5.1.2 Sporulation

Sporulation was studied according to whether the isolates showed spores under the microscope in order to determine whether the sporulation of isolates was influenced by the increase in fluopyram concentration and their field treatments (Propulse vs. untreated).

If an isolate showed spores as in Fig. 2, sporulation was noted, if no spores were visible then it was assessed that there was no sporulation.

The data was analysed with a generalized linear model Anova with the dependent variable the sporulation or not and involving the fixed factors fluopyram concentrations and their field treatment. Because of its phenotype, isolate N20.2.3 was not included in the analysis.

2.5.2 Soil data analysis

2.5.2.1 Weight of bags

The weight difference of the bags before and after soil trial was calculated by subtracting the weights after the inoculation (after) from the weights before the inoculation (before).

In Anova analysis the weight difference was used as the dependent variable in testing the effect of the fixed factors: farm, soil treatment and their interaction.

2.5.2.2 qPCR

The Ct values obtained for the standards were used to construct a standard curve. A mean of the Ct was plotted against the log of the DNA concentration using linear regression model. The DNA concentration of the samples was calculated based on the equation of the linear regression curve. The exponential of the logarithmic value was calculated to obtain the DNA concentration of the samples. The PCR efficiency was calculated using the formula $E = (10^{(-1/\text{slope})} - 1) \times 100$ as described in Kumar *et al.* (2013).

The qPCR results were then analysed in a mixed Anova with the dependent variable DNA concentration (log-transformed) and the fixed factors soil type, soil condition, their interaction. The random factor farm was included to control for repeated measures of the farms.

Results

3.1 *Alternaria solani* isolates

Leaves with *A. solani* lesions where collected in potato field trials in Nymö and Lyngby gård, Sweden, in years 2018 - 2022. Both leaves from field plots treated with the newly introduced fungicide Propulse and untreated plots were collected. *Alternaria solani* was isolated from the collected leaves using single cell isolation method. From all the samples, 30 isolates were selected for further analysis, and 25 were used, due to failure to cultivate all 30 of them.

3.2 Selection of fluopyram concentrations for the growth assays

Sensitivity to fluopyram, a fungal growth inhibitor, was tested with liquid and solid growth assays. The tested samples were grown in presence of various concentrations of the drug in order to determine the range of the fluopyram concentrations. We designed a try-out growth assay, where we grew the lab strain (As112) in liquid PDB medium with fluopyram concentrations ranging from 0 to 20 µg/ml. The mycelial growth was measured after 7 days and the obtained data was used to construct a growth curve (Fig 5.).
The growth had a clear decrease when we increased the fluopyram concentration. The lowest concentration that was lethal for As112 was 15 µg/ml, which became our highest concentration for the growth assays with field isolates.The selected concentrations were 0.5 µg/ml; 0.958 µg/ml; 2.66 µg/ml; 4.13 µg/ml; 7.05 µg/ml and 15.0 µg/ml.

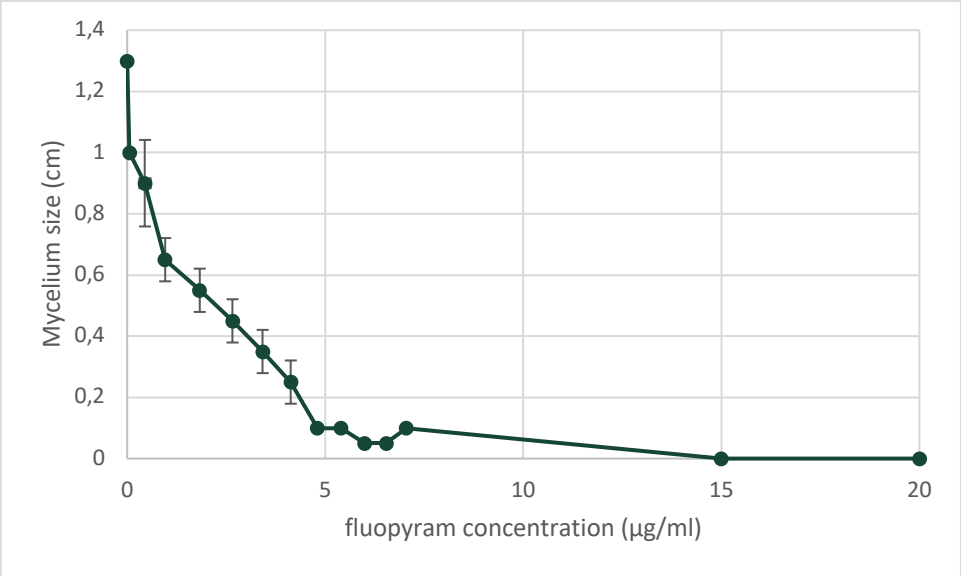


Figure 5. Growth try-out for A. solani lab strain (As112) for 15 different fluopyram concentrations

3.3 Liquid growth assay with field isolates

Growth assays were performed to assess fluopyram efficacy and the resistance development of the different field isolates. The 25 isolates were grown in PDB medium mixed with different concentrations of fluopyram. Two liquid assays were performed, the first one with the 25 isolates and the second one with 14 isolates selected among the 25 isolates. In both of the liquid assays, the *A. solani* isolates showed a decline in the growth when the concentration of fluopyram increased (Fig 6. , Tab 2.). A Post-hoc test showed that growth for the concentrations C6, C5 and C0 were all different from each other and different from C3-C1. C3-C1 were not different from each other. The effect of the concentration was different over the years, as suggested by the significant interaction between year and concentration (Tab. 2). The reduction of growth between the C5 and C0 concentrations was highest in 2018 and lowest in 2021 and 2022 (Fig. 7), indicating increased tolerance to fluopyram in later years.

One of the 2020 isolates did not respond to the different concentrations at all (Fig 6C.), this isolate was also phenotypically different from the others isolates as it was white.

The fungicides treatments applied in the fields did not influence the growth of the isolates, as we did not see a difference between the growth of the treated and the untreated isolates (Tab. 2, Fig. 6, 7.).

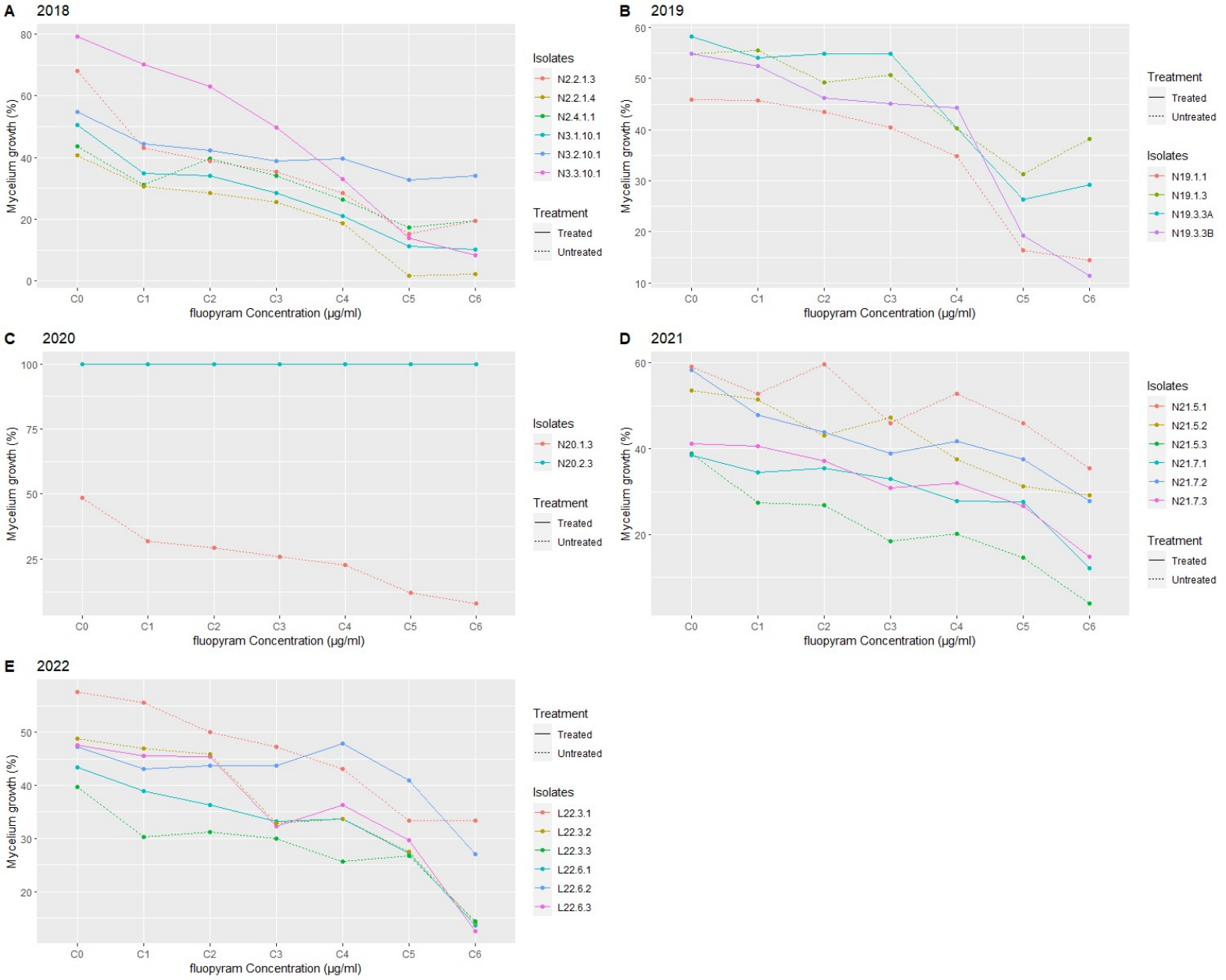


Figure 6. Liquid growth assay of A. solani isolates collected in years 2018-2022. The figures (A to E) show percentage growth of mycelia of the different isolates in seven concentrations of fluopyram (C0-C6). Solid or dotted lines indicate if the samples were collected from plots treated with fungicides or not

Table 2. Mixed model Anova analysis for the growth of A. solani in liquid assays as an effect of collection year (excluding 2020), fluopyram concentration, treatment with fungicides vs untreated and their interactions. Isolate ID was included in the model as a random factor. F-value, P-value and degrees of freedom (DF) are presented for each factor and their combinations.

Source of variation	F-value	DF	P-value
Year	0.62	3, 14	0.61
Treatment	0.27	1, 14	0.61
Concentration	84.9	6, 84	<< 0.0001
Year:Treatment	0.50	3, 14	0.69
Year:Concentration	3.03	18, 84	0.00031
Treatment:Concentration	0.61	6, 84	0.72
Year:Treatment:Concentration	0.38	18, 84	0.99

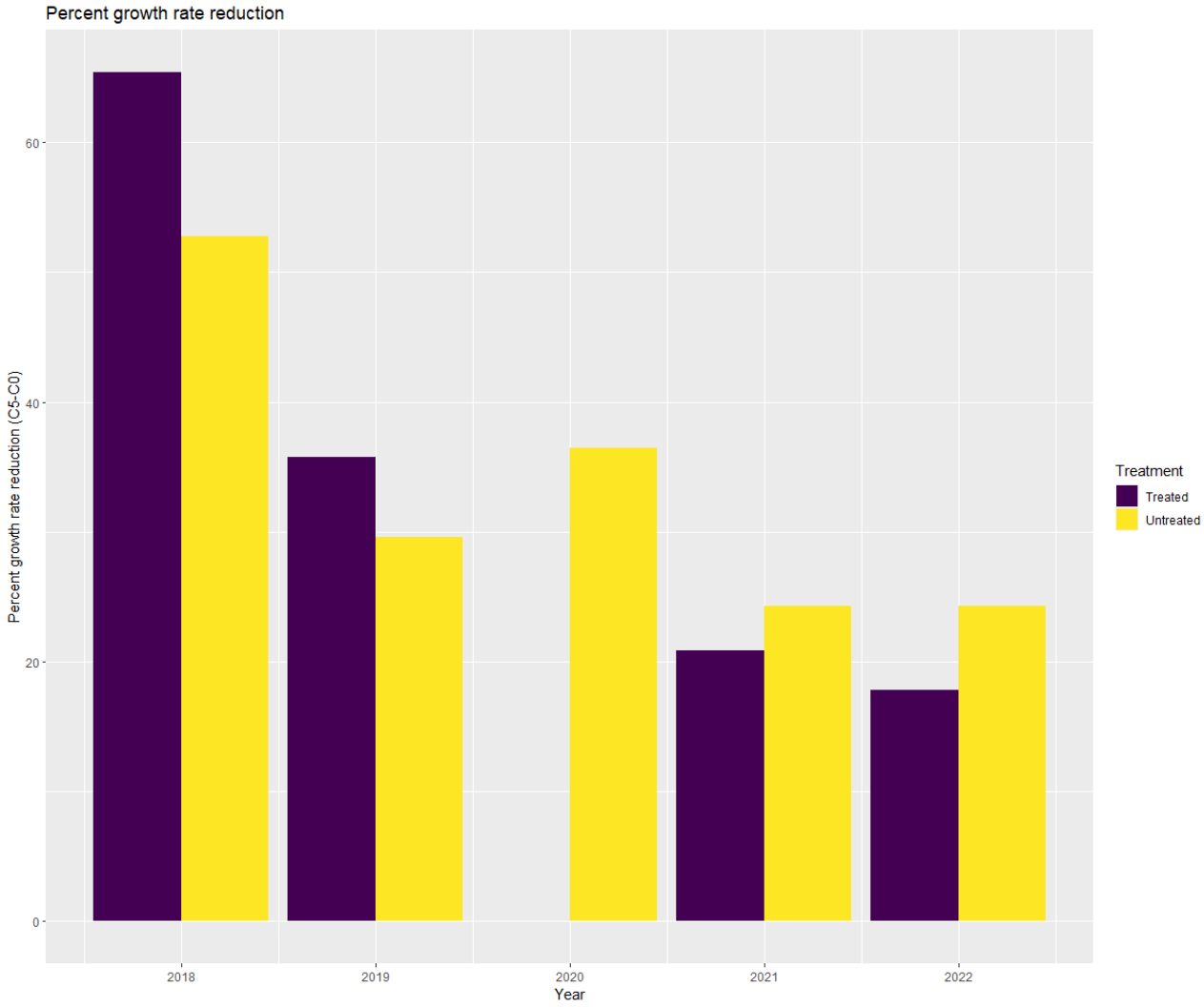


Figure 7. Percent growth rate reduction for A. solani isolates collected in years 2018-2022. The figure shows the rate of reduction for the growth of mycelia of the different isolates in the seven concentrations of fluopyram (C5-C0). The different isolates came from Nymö for the years 2018-2021 and Lyngby gård for 2022.

3.4 Solid growth assay with the field isolates

The 14 previously selected isolates were used again for the solid assay. From the previous 25 isolates, 14 were selected according to the results of the liquid assay. The selected isolates were grown on 20% PDA plates with the same fluopyram concentrations as for the liquid assay. This growth assay was performed to analyse the growth patterns of the selected isolates on solid media, but also to assess their sporulation ability.

The solid growth assays showed a decline in the growth when the concentration of fluopyram increased (Fig. 8, Tab. 3). This decline was, however, not as severe as in the liquid assays and the majority of the cultures showed a slight size increase in the lowest concentration (C1) compared to the control solution (C0) (Fig. 8). Although the growth patterns varied between the isolates and years, a post-hoc test showed that the size of the mycelium was smaller in the highest fluopyram concentration (C6) compared to all the other concentrations, C5 to C1 were not different from each other, and C4 to C0 were not different from each other. In contrast to the liquid assay, the concentration by year interaction was not significant (Tab. 3). There was, however, a marginally non-significant interaction between year, concentration and treatment (p-value = 0.06) (Tab. 3). The treatment alone did not affect the growth significantly as is suggested by the p-value in table 3. Isolate N20.2.3 was also white like in the liquid assay, but responded to the different fluopyram concentrations (Fig 8 & 9).

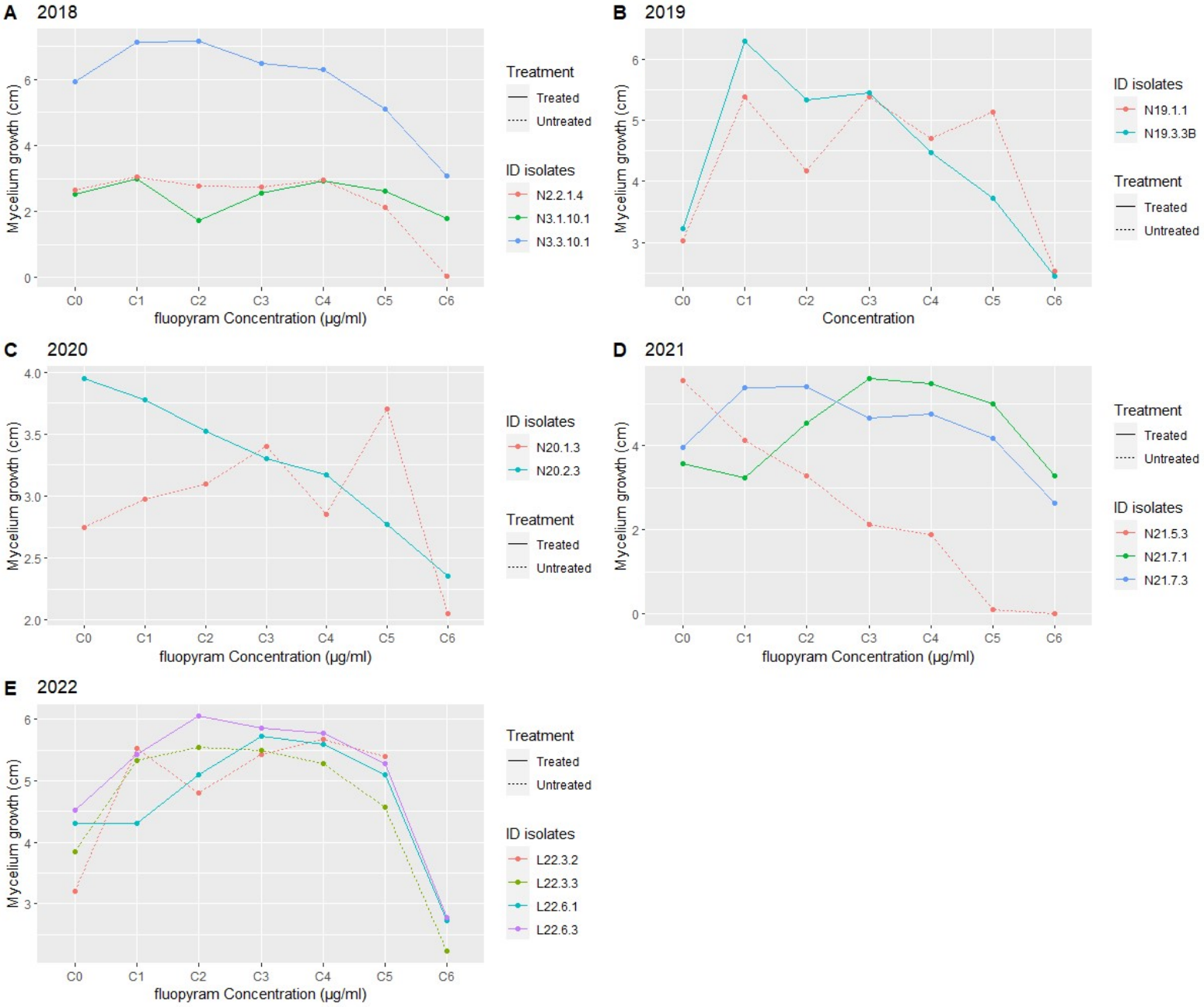


Figure 8. Solid growth assay of A. solani isolates collected in years 2018-2022. The figures (A to E) show mean growth of mycelia of the different isolates in the seven concentrations of fluopyram (C0-C6). Solid or dotted lines indicate if the samples where collected from plot treated with fungicides or not.

Table 3. Mixed model Anova analysis for the growth of A. solani in solid assays as an effect of collection year (excluding 2020), fluopyram concentration, treatment with fungicides vs untreated and their interactions. Isolate ID was included in the model as a random factor. F-value, P-value and degrees of freedom (DF) are presented for each factor and their combinations.

Source of variation	F-value	DF	P-value
Year	0.94	3, 3.99	0.52
Treatment	1.79	1, 3.99	0.25
Concentration	20.80	6, 23.00	<< 0.0001
Year:Treatment	0.37	3, 3.99	0.82
Year:Concentration	1.45	18, 23.00	0.19
Treatment:Concentration	0.65	6, 23.00	0.69
Year:Treatment:Concentration	1.91	18, 23.00	0.06

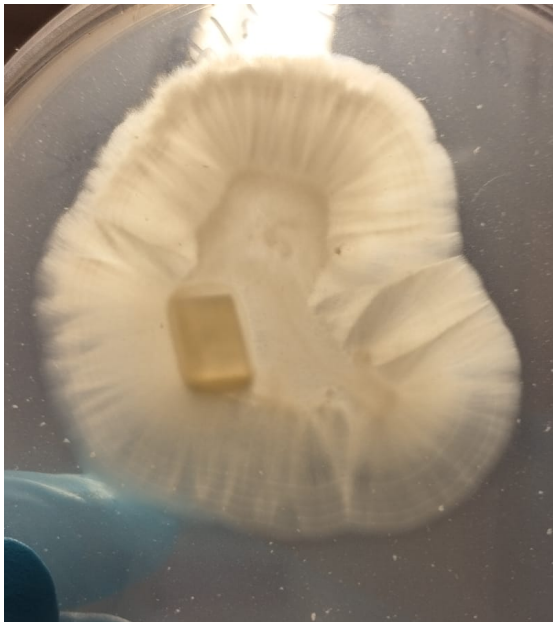


Figure 9. N20.2.3 isolate growth on a 20% PDA plate.

3.4.1 Validation of *A. solani* isolates

To confirm that the 14 isolates investigated in the growth assays were *A. solani* we extracted genomic DNA from the mycelia and performed a PCR analysis with species-specific primers. NanoDrop analyses were performed to assess the DNA concentration and quality in our samples. High quality gDNA was extracted from all isolates (Tab 4.). The 260/280 and 260/230 ratios show the purity of our samples, both should be around 2.0 in high quality samples. The first ratio indicates possible contamination with organic solvents, while the second ratio, 260/230, indicates the presence of protein and/or salt contamination. Most of our samples were pure,

however N21.7.1, N21.7.1, L22.3.3, L22.6.1, L22.6.3 samples had 230/260 ratios much below the expected 2.0, indicating potential protein or salt contamination (Tab. 4).

The PCR results (Fig 10.) confirm that all our isolates were *A. solani*. All PCR product bands were the same size of about 250bp, which corresponds to the expected product. For isolate N20.2.2 we observed two weaker bands of about 700 bp and 2000 bp, suggesting unspecific amplification. Since the primers were verified to be specific to the β-tubulin gene and to *A. solani* these bands might be indicative of DNA degradation in the sample.

The analysis confirmed that the isolate N20.2.3 that displayed a different colour than the others, was indeed *A. solani* (Fig 10.).

Table 4. DNA quantification with NanoDrop Specrometer of the 14 A. solani isolates used for the solid assay and liquid assay.

sample	*concentration [ng/ul]*	*260/280*	*260/230*
N3.3.10.1	144.70	1.97	1.85
N3.1.10.1	162.07	1.97	2.02
N2.2.1.4	156.99	1.98	2.09
N19.1.1	225.60	1.97	2.12
N19.3.3B	247.18	1.96	2.22
N20.2.3	126.50	1.93	1.89
N20.1.3	93.09	2.03	2.12
N21.7.3	122.35	1.99	1.80
N21.7.1	110.29	1.96	1.53
N21.5.3	135.10	1.97	1.66
L22.3.2	191.91	1.95	2.00
L22.3.3	128.09	1.98	1.42
L22.6.1	77.88	1.98	1.66
L22.6.3	96.67	1.99	0.82

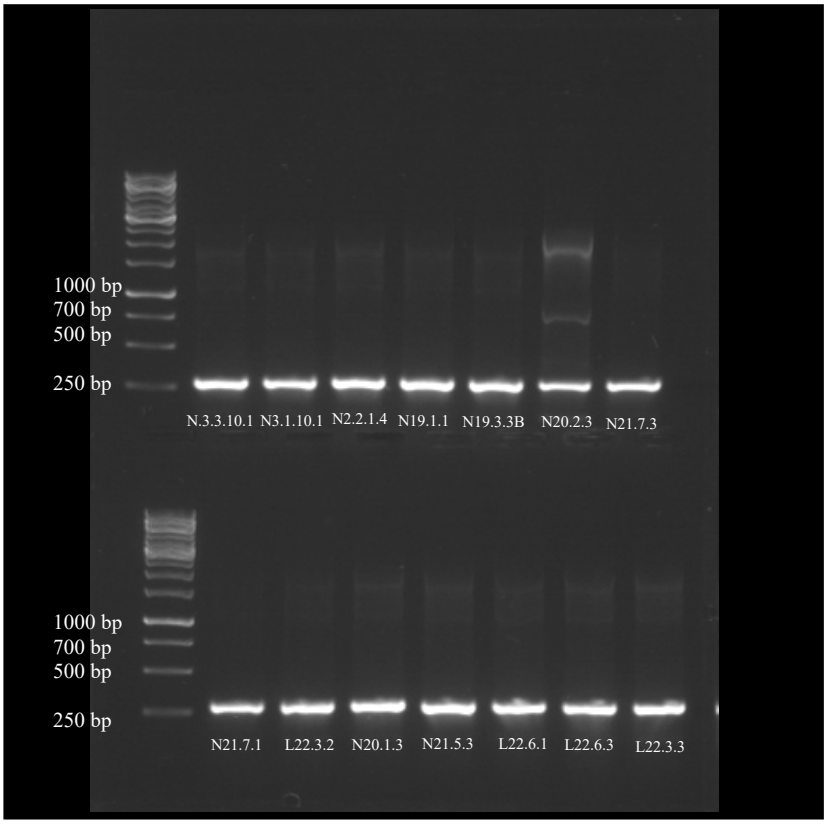


Figure 10. Gel electrophoresis of the species-specific PCR performed on 14 isolates of A. solani collected in years 2018-2022.

3.4.2 Sporulation assessment from the solid assay isolates

The isolates that were grown on solid media were assessed for the sporulation ability. After incubation in the near-UV incubator which induces sporulation, the plates where looked at under a stereo microscope and the sporulation was assessed (Fig 11.).

The sporulation differed for all the years and isolates. Overall, the isolates did not had less sporulation with an increase in fluopyram concentration (Appendix 1). However the concentration had a significant effect (Tab 5).The year from which all the isolates seem to be the most affected by the concentrations is the year 2018. The concentrations with the highest number of non-sporulated isolates are C5 and C6, with two that did not sporulate. For all concentrations, except for C3, there were some isolates that did not sporulate. For C6 there were 5 isolates that did not sporulate. For the years 2019, 2020, and 2022 all isolates sporulated and for 2021 there was one isolate that did not sporulate (Fig 12.). The previously applied fungicide treatment did not have a significant influence on the isolates' ability to sporulate (Tab 5.).

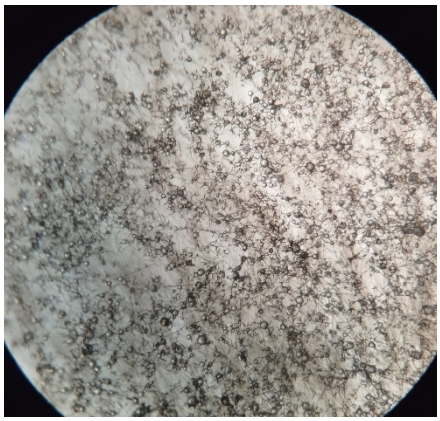
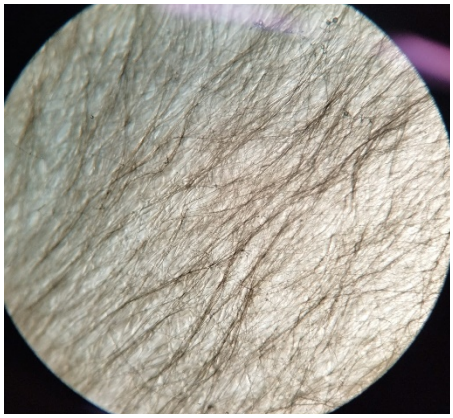
sporulating mycelium	*Mycelium without spores*
	

Figure 11. A. solani cultures on solid media. A. sporulating mycelium, B. non-sporulating mycelium; as seen under a stereo microscope, magnification 100x.

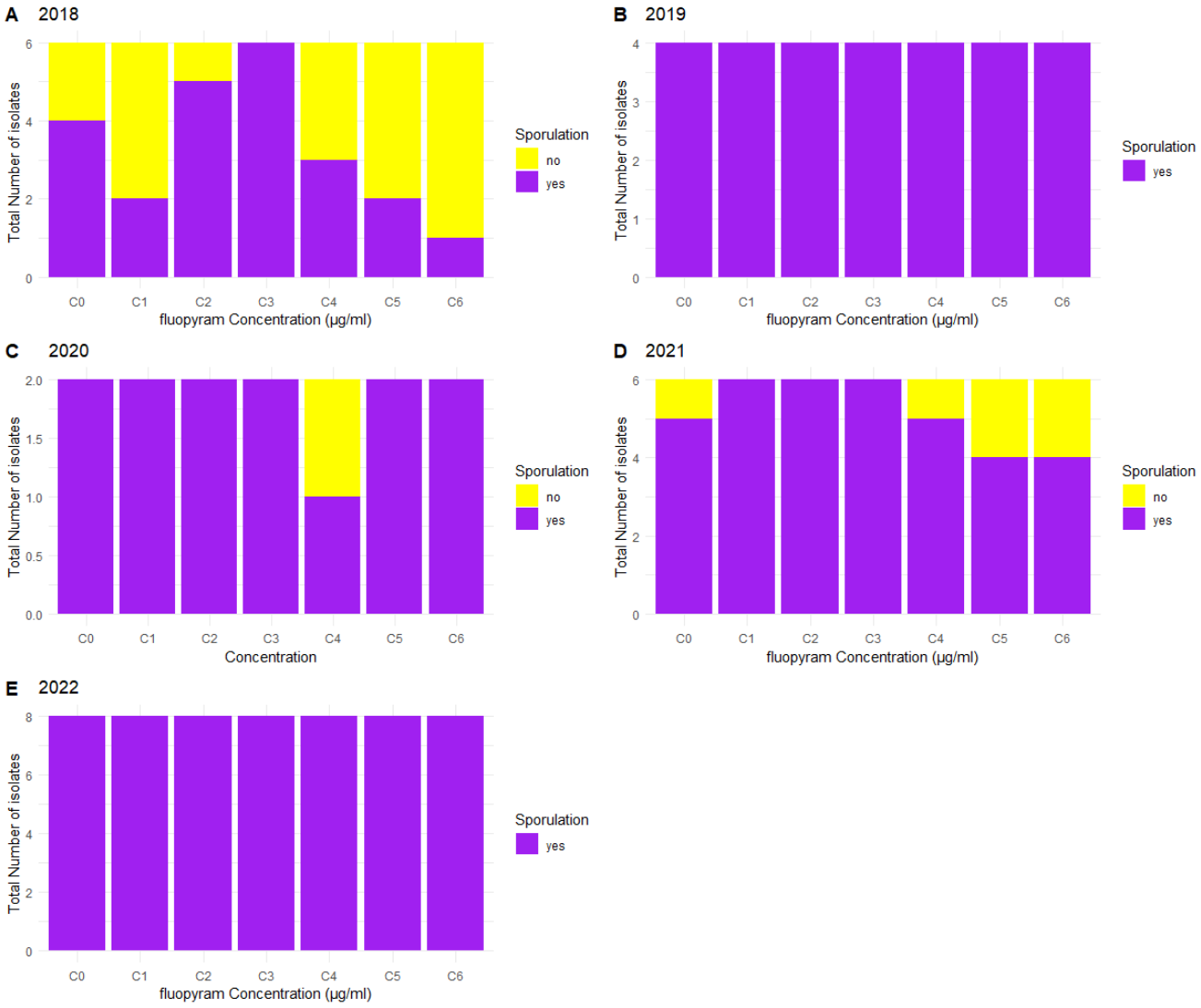


Figure 12. Sporulation of A. solani isolates collected in years 2018-2022. Isolates from plots treated with fungicides vs. untreated isolates are merged in this figure. The figures (A to E) show sporulation or its lack in the different isolates in the seven concentrations of fluopyram (C0-C6).The purple colour shows the number of isolates that sporulated, while the yellow bars represent non-sporulating isolates.

Table 5. Mixed model Anova analysis for the sporulation of Alternaria solani in solid assays as fluopyram concentration, treatment with fungicides vs untreated and their interactions. F-value, P-value and degrees of freedom (DF) are presented for each factor and their combinations.

Source of variation	F-value	DF	P-value
Treatment	1.83	1	0.18
Concentration	2.83	6	0.01

3.5 *A. solani* survival in the soil depending on the soil composition and condition

For the soil assay, we inoculated rye kernels with As112, *A. solani* lab strain, and put them in four different soils from different fields for 66 days. The four soils were divided in two types : sandy or clay. For each soil there were three different conditions : sterilized, unsterilized wet or unsterilized dry. The infected rye was weighed before and after the incubation in the soil, then a qPCR was performed in order to quantify *A. solani* in the different samples.

3.5.1 Soil type and nutrient analysis

From the analysis of the soils of the potato farm fields used as a source for the soil assays (Tab. 2) we classified two as sandy, PET and VIK (sand content 19% and 12%, respectively). The two others, HAK and JAR, were classified as clay soils (sand content 5.6% and 3.5%, respectively) (Tab 6.).
The phosphorus content was higher in the clay soils, however PET had a higher phosphorus content than VIK and only slightly lower than the other ones.
The potassium content was more variable and the soil with the highest content in potassium was JAR (16 mg/100g) then it was PET, followed closely by VIK and then HAK with 3.6 mg/100g (Tab 6.).

Table 6. Soil type and nutrients (phosphorus and potassium) content of the four potato fields used as a source for the soil assay on survival of Alternaria solani. Data extracted from Eurofins rapport (Appendix 1)

Field	P-Al mg/100g	K-Al mg/100g	Sand/coarse soil (%)	Clay content (%)
Pet	17	12	19	46
Vik	9.3	10	12	60
Hak	21	3.6	3.5	88
Jar	27	16	5.6	74

3.5.2 Inoculated rye kernels from the soils

In order to investigate the effect of soil composition (see previous section) and condition (unsterilized wet or dry and sterilized) on the survival of *A. solani*, bags with inoculated rye seeds were dug into pots of four different soils (HAK, PET, JAR, and VIK). All bags with rye kernels were weighed before and after the inoculation in order for the bags to be the same weight across the whole experiment. For all the bags under all conditions, the bags after the incubation time were lighter than previously (Fig 13 & 14.). The conditions were significant, as well as the farms and the relationship between them (Tab 7.). Among the conditions for HAK (3.6% of sand content), a post-hoc test showed that the only significant difference was between the sterilized condition and the two other (Fig 13.). For PET (19% of sand content), the dry condition was the one which was significantly different from the two other (Fig 13.).

Under the dry condition, the soil with the smallest difference in bags' weight was HAK (Fig 14.). Also the difference between HAK (3.5 % of sand content) and the three other farms was significant (Fig 14.). For the sterilized condition all soils had more or less the same weight (Fig 14.). For the unsterilized wet condition HAK had the heaviest bags (39,17g) of all the soils (Fig 14).

For the difference between the weight before and after incubation of the infected rye kernels in the soil, the dry condition had the smallest overall difference (Fig 14.). For HAK the condition that had the smallest difference was the unsterilized wet one. For JAR, PET and VIK it was unsterilized dry. The condition with the largest difference is the sterilized one (Fig 14).

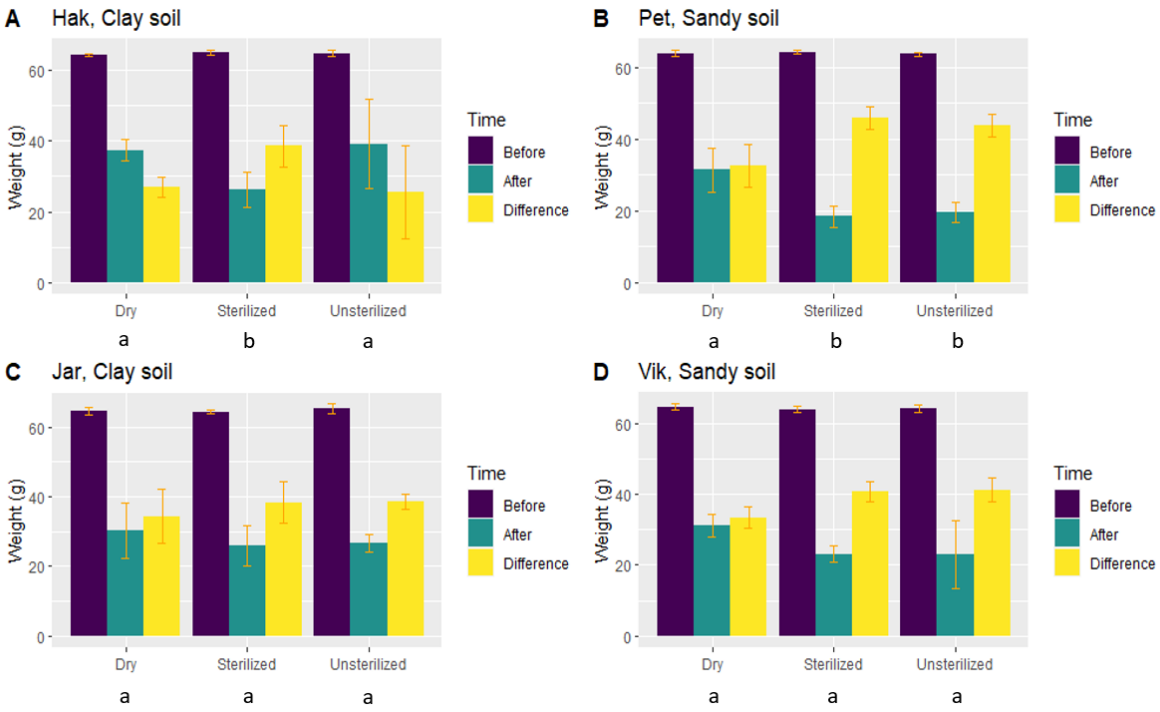


Figure 13. Mean weight of A. solani- infected kernels before and after incubation in four different soil types. The figures (A to D) show the weight for the four different soils (HAK, PET,JAR and VIK) and the three different conditions (unsterilized dry, unsterilized wet or sterilized). Different letters refer to significant ($P < 0.05$) differences between weight differences.

Table 7. Mixed model Anova analysis for weight of the A. solani-inoculated kernels in soil assays as farms ,unsterilized dry, unsterilized wet and sterilized, and their interactions. F-value, P-value and degrees of freedom (DF) are presented for each factor and their combinations

Source of variation	F-value	DF	P-value
Farm	12.29	3	<< 0.0001
Condition	15.88	2	<< 0.0001
Farm:Condition	2.81	6	0.017

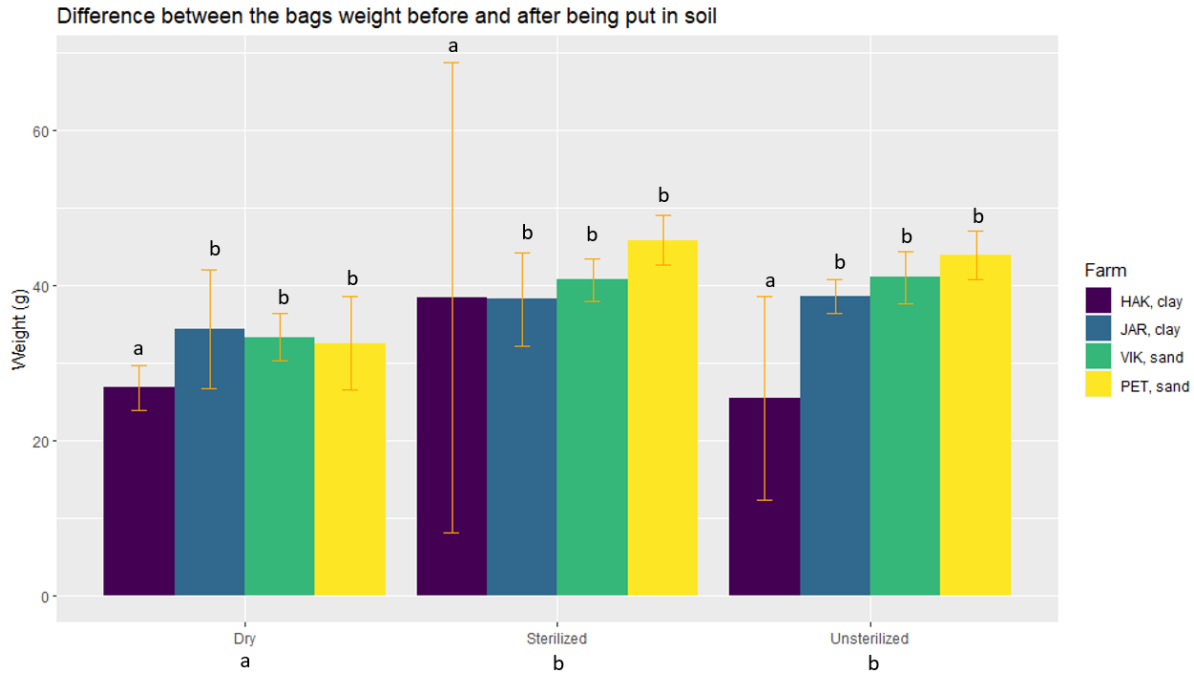


Figure 14. Difference between the rye kernel weight before and after the incubation in sandy or clay soils (HAK, PET, JAR, and VIK) for the three different condition (unsterilized dry, unsterilized wetand sterilized). The different colours represent the four different farms and the type of soil. Different letters refer to significant ($P < 0.05$) differences between the farms and the soil conditions.

Figure 15. A bag with infected kernels and visible white mycelium, after nine week incubation in soil.

3.5.3 DNA extraction from the infected kernels

From all the 84 bags of kernels used for the soil assay, 50 were selected according to the type of soils (HAK, PET, JAR or VIK) and the condition (dry unsterilized, sterilized or wet unsterilized, referred in the figures respectively as dry, sterilized or unsterilized) they were incubated in. The extraction of genomic DNA from the infected kernels proved challenging and both the quantity and quality of the DNA were lower than expected. Particularly the 230/260 ratios were low. Therefore, the samples were purified with Sephadex™ G-50 DNA grade resin. Although the filtration through de-salting columns increased both the concentration and purity of the samples, the quality was still much worse than desired (Tab 8.).

Table 8. DNA quantification with Nanodrop for 50 samples after DNA extraction and purification.

sample	concentration [ng/ul]	260/280	260/230
Vlk.U.1	314.85	1.77	1.33
Vlk.U.3	240.91	1.73	1.16
Vlk.U.5	277.91	1.76	1.21
Vlk.U.6	221.71	1.69	1.03
Vlk.S.1	589.87	1.88	1.95
Vlk.S.2	180.45	1.70	1.03
Vlk.S.5	60.20	1.33	0.47
Vlk.S.6	217.10	1.70	1.12
Vlk.D.3	170.20	1.70	1.06
Vlk.D.4	71.52	1.30	0.51
Vlk.D.6	137.32	1.61	0.90
Vlk.D. 7	48.31	1.22	0.46
Vik.D.5	60.78	1.29	0.58
PET.D.1	113.88	1.51	0.77
PET.D.5	145.73	1.66	0.98
PET.D.6	82.62	1.46	0.66
PET.S.1	253.19	1.72	1.19
PET.S.2	127.69	1.59	0.84
PET.U.1	129.88	1.62	0.83
PET.U.3	136.21	1.52	0.79
PET.U.5	353.39	1.80	1.38
PET.U.6	103.75	1.51	0.71
JAR.S.1	273.81	1.77	1.27
JAR.S.2	268.19	1.75	1.26
JAR.S.3	201.60	1.70	1.10
JAR.S.5	60.95	1.32	0.50
JAR.S.6	101.00	1.55	0.73

JAR.D.2	146.18	1.68	0.98
JAR.D.3	49.7	1.30	0.48
JAR.D.4	68.11	1.44	0.62
JAR.D.6	158.85	1.64	1.07
JAR.D.7	256.08	1.76	1.26
JAR.U.2	350.16	1.78	1.40
JAR.U.5	208.94	1.72	1.10
JAR.U.6	194.11	1.71	1.12
JAR.U.7	337.48	1.80	1.40
HAK.D.1	236.02	1.74	1.26
HAK.D.2	86.02	1.51	0.66
HAK.D.3	37.55	1.06	0.35
HAK.D.4	26.02	0.96	0.25
HAK.D.6	116.06	1.58	0.81
HAK.D.7	23.05	1.73	0.69
HAK.S.1	344.02	1.80	1.39
HAK.S.3	205.13	1.70	1.05
HAK.S.5	277.35	1.75	1.23
HAK.S.6	491.64	1.79	1.47
HAK.U.1	425.82	1.80	1.41
HAK.U.2	470.8	1.81	1.53
HAK.U.4	507.21	1.80	1.52
HAK.U.7	412.84	1.80	1.46

3.5.4 qPCR on the inoculated kernels

The qPCR analysis was performed to estimate *A. solani* DNA concentration in each sample. The samples were divided between two qPCR plates, as we had too many samples for one, and therefore there was a separate standard curve constructed for each plate. The qPCR efficiency for the first analysis was 86.77% and for the second it was 77.06%. The expected optimal efficiency is between 90% - 100%, our qPCR had lower efficiency. All our sample results fall on the linear regression of both of the standard curves, except the samples that had a Ct-values over 38, which should be disregarded regardless.

Twelve *A. solani* samples yielded Ct-values above 36 and were disregarded due to low credibility (Tab 9.). Two samples, VIK.U.5 and HAK.U.1 yielded Ct values of zero, which indicates problems with fluorescence measurement, most likely due to poor DNA quality and thus the DNA concentration was not calculated for these samples(Tab 9.) There was a significant effect of the soil condition on the DNA concentration but not of the type of soil (Tab 10.). The conditions had a different response for each farm (Fig 18.) , but the type of soil did not have an influence on the DNA concentration (Tab 10.). A post-hoc test showed that for both types of soil (sandy and clay), the dry condition is different than the sterilized or unsterilized

condition. The qPCR results showed that the dry condition had higher DNA concentration than the two other conditions for both sand and clay soils (Fig 18.).

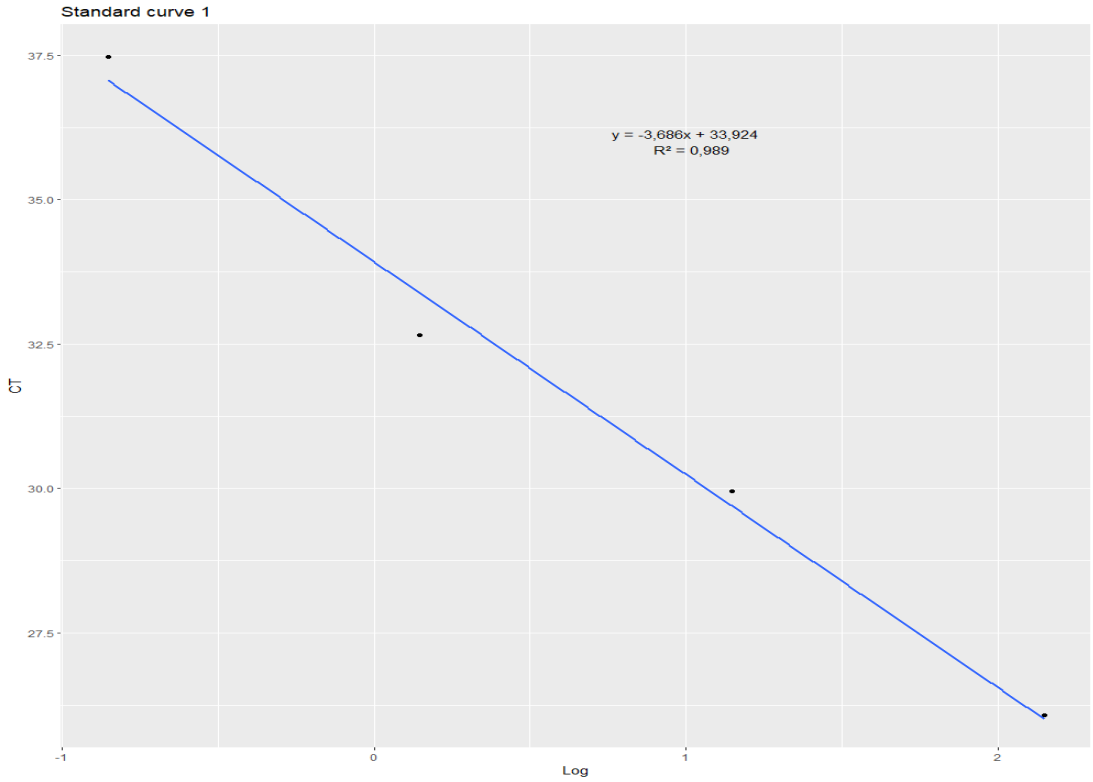


Figure 16. Standard curve obtained from the qPCR results for lab strain As112. The DNA concentration was known for the samples, which we used to calculate their log, the x-axis.

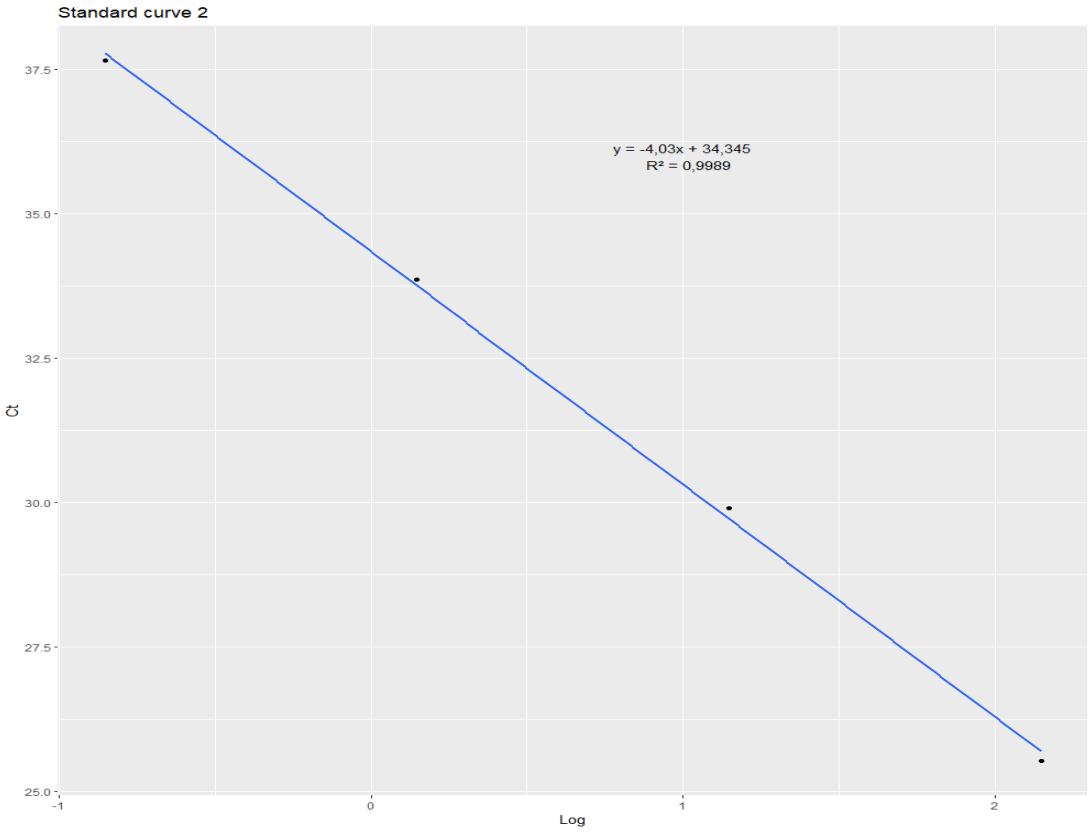


Figure 17. Standard curve obtained from the qPCR results for lab strain As112. The DNA concentration was known for the samples, which we used to calculate their log, the x-axis.

Table 9. DNA concentration of the infected kernel samples, calculated with the exponential of the log of the result of the linear regression from the respective standard curve (1 or 2), where x is the Ct obtained from the qPCR. Asterisk indicates missing values.

Sample	CT	DNA concentration (µg/ml)	Standard curve
VIK.U.1	36,3836662	0,21512973	1
VIK.U.3	32,7787741	2,04501317	1
VIK.U.5	0	*	1
VIK.U.6	33,4429757	1,35051791	1
VIK.S.1	36,1590595	0,24753437	1
VIK.S.2	34,8555839	0,55881011	1
VIK.S.5	33,7056489	1,14614076	1
VIK.S.6	39,4909032	0,03088282	1
VIKD.D.3	29,2132923	18,9670562	1
VIK.D.4	30,9970641	6,22398198	1
VIK.D.6	28,4213489	31,1065338	1
VIK.D.7	31,1051327	5,81767779	1
VIK.D.5	29,8848825	12,4680822	1
PET.D.1	28,4859995	29,8752906	1
PET.D.5	32,2582871	2,83075692	1
PET.D.6	30,6511373	7,72533237	1
PET.S.1	33,9276576	0,99771777	1
PET.S.4	36,0988478	0,25702228	1
PET.U.1	33,3438723	1,43676847	1
PET.U.3	35,9491232	0,28222181	1
PET.U.5	39,9866232	0,02265843	1

PET.U.6	36,6878795	0,17789688	1
JAR.S.1	34,891167	0,54652584	1
JAR.S.2	34,9375975	0,53090186	1
JAR.S.3	32,9389228	1,85032615	1
JAR.S.5	33,0470155	2,09932904	2
JAR.S.6	33,1306218	2,00140297	2
JAR.D.2	27,5008456	49,9230761	2
JAR.D.3	32,2677235	3,2768249	2
JAR.D4	28,2868565	31,8611484	2
JAR.D.6	28,9497729	21,8155428	2
JAR.D.7	26,3828646	94,561852	2
JAR.U.2	38,3275363	0,102749	2
JAR.U.5	38,2359407	0,10826947	2
JAR.U.6	34,6693685	0,83082881	2
JAR.U.7	29,7568924	13,7558753	2
HAK.D.1	29,0984184	20,0392417	2
HAK.D.2	29,9572977	12,2676078	2
HAK.D.6	27,3791189	53,5188122	2
HAK.S.1	34,6795638	0,8260031	2
HAK.S.3	35,3730148	0,55578839	2
HAK.S.5	36,2110018	0,34432793	2
HAK.S.6	36,2562613	0,33553791	2
HAK.U.1	0	*	2
HAK.U.2	39,8474221	0,04311563	2
HAK.U.4	36,9486149	0,22591326	2
HAK.U.7	33,9710562	1,23819778	2

Table 10. Mixed model Anova analysis for the DNA concentration for A. solani-inoculated kernels in soil assays as type of soil (clay or sand) ,dry, sterilized or unsterilized conditons and their interactions. F-value, P-value and degrees of freedom (DF) are presented for each factor and their combinations.

	F-value	DF	P-value
Type of soil	2.61	1, 1.17	0.27
Condition	43.19	2, 35.45	<< 0.0001
Type of soil:Condition	0.62	2, 35.44	0.54

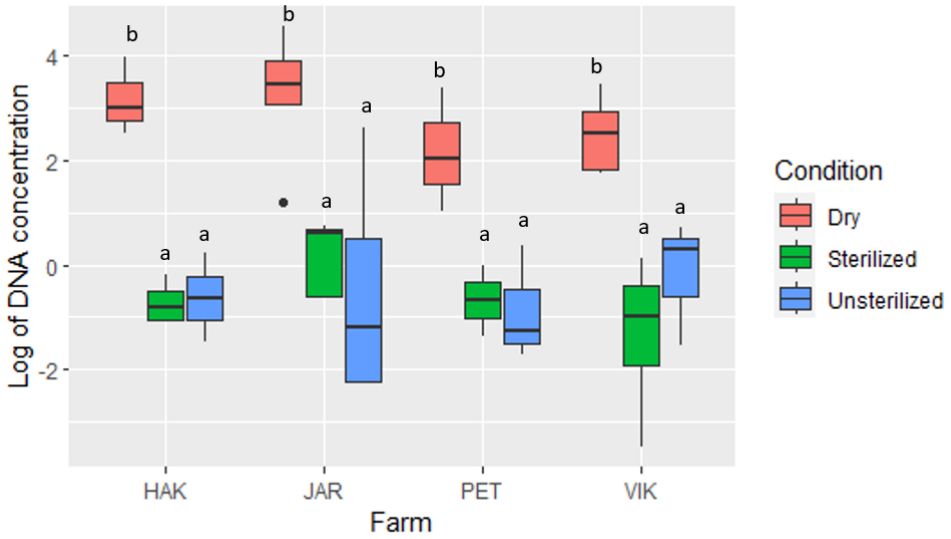


Figure 18. Log of DNA concentration obtained through a qPCR from the inoculated kernels in HAK, PET, VIK, and JAR. The three different conditions with which the soils were treated were: Dry, Sterilized, or Unsterilized. Different letters refer to significant ($P < 0.05$) differences between the conditions.

DISCUSSION

4.1 Growth assays

The first aim of this study was to assess the effect of fluopyram on *A. solani* growth and sporulation, and to see if previous fungicide treatments in the field had an effect on the pathogen's sensitivity to the drug. The fluopyram concentrations were decided based on a try-out where we tested a lot of different concentrations of the drug (up till 20 µg/ml) to determine the range of concentrations effective on the growth of the lab strain As112. At 0,958 µg/ml mycelial growth was reduced by half and the lethal concentration, i.e. no growth at all, was 15 µg/ml. That is why we selected these two concentrations for assays with the field isolates. Additionally, we selected a range of lower concentrations in order to study the effect of small doses of fluopyram on the growth of *A. solani*.

While the general trend in both of our growth assays was decreased mycelial growth with the increased fluopyram concentration, there were some exceptions, particularly in the liquid growth assay. What is more, we observed different results for the same isolates in the two assays. The most interesting, but at the same time puzzling, isolate was N20.2.3 which turned white and did not respond to any of the fluopyram concentrations in the liquid assay (two replicates per concentration in two separate assays), yet had a steep growth decrease with increasing drug concentrations on solid plates. The mycelium size decrease was not linear, as would be expected, for most of the isolates. In fact, in the solid growth assay a lot of isolates had a larger size in lowest fluopyram concentration (C1) than in the control (C0). These results could indicate that very low fluopyram concentrations do not influence the growth of the isolates, thus it is possible that a threshold exists for fluopyram to be effective on the field isolates. The previously applied treatment (i.e. fungicides sprayed in the field) did not affect the growth of our isolates, and was not a significant factor in their growth. Nevertheless, the lack of visible and significant effect on the growth does not exclude the possibility of partial resistance build-up, which could explain the size increase between the control and C1 concentration in several of the investigated isolates.

The highest fluopyram concentration tested, C6, proved to inhibit fungal growth in all tested isolates thus showing that although the sensitivity to the drug varied between the samples, all responded to the treatment. Given that in the fungicide Propulse the concentration of fluopyram is 125 g/l (Bayer Crop Science b) which is 8 times more than C6 solution, our study showed that it could be possible to reduce the concentration of fluopyram in the fungicide, and it still would be effective. As said in the introduction, Propulse is toxic to the aquatic life, so such decrease in the effective dose is highly desirable.

The sporulation of the cultures seems to not be affected by the different concentrations of fluopyram, but rather by the isolate itself. The only exception was year 2018 which seems to have more isolates that did not sporulate under high concentrations. But in general, the increase in fluopyram concentration does not seem to influence the sporulation, rather the sporulation of the isolates seems to be random and more related to the year of collection. It means that even though the concentration of fluopyram may affect the size and rate of the mycelium growth, the sporulation ability of a specific isolate depends on other factors.

Importantly, the growth also seems to be less sensitive to fluopyram in the later years than in the earliest year. It may imply that in the later years (2019-2022) the pathogen strains have already been exposed to fluopyram before, and became less sensitive. Which is consistent with our hypothesis that fluopyram resistance is building up is some of our isolates, but does not manifest clearly in the growth patterns. It is also possible that the isolates are developing tolerance to fluopyram rather than resistance. However *A. solani* insensitivity development to fluopyram due to mutations have been reported (Bauske et al., 2018).

All isolates reacted differently to fluopyram, and we could see that some isolates were more affected by the high concentrations than others, regardless of the collection year or previous treatment. It could suggest that the resistance/tolerance development is unique to each isolate, and does not depend on previous factors such as treatment. The development of different levels of resistance to boscalid fungicide have been reported before, and individual sensitivity may differ for different fungicides (Gudmestad et al., 2013).

PCR analysis with *A. solani* specific primers confirmed that all fourteen isolates selected in the growth assays were indeed *A. solani*, including the white isolate which raised some suspicion due to its phenotype, but also lack of sensitivity to fluopyram in the liquid growth assay. The PCR product of this isolate displayed weak bands additional to the bright and clear β-tubulin band on the electrophoresis gel. This could be because of overloading of the wells, spilling some PCR product outside or too high initial DNA concentration. However, it could also mean that our DNA sample was degraded. Nevertheless, this result indicates the possibility of a rapid phenotypical change of *A. solani.* The isolate original plate did exhibit a typical *A. solani* growth which is dark brown/black mycelium growing in alternated ring pattern, and the white phenotype appeared after the re-culturing of this plate.

This isolate did not present visible spores, even under the control condition. Still it could be possible that the spores are not visible with naked eyes. Both the phenotypical changes and the results of the liquid growth assay make this isolate a very interesting candidate for sequencing in future studies.

In the future studies it would also be interesting to investigate *A. solani* growth in fluopyram concentrations between C5 and C6, but also above C6. The C5 fluopyram concentration is 7.05 µg/ml and C6 is 15 µg/ml, which means that there are a lot of possible concentrations to test in between. As our highest concentration (15µg/ml) had a clear effect on mycelia growth of all isolates, it would be interesting to test a wider range of fluopyram between C5 (7.05 µg/ml) and C6 (15µg/ml), to investigate if lower concentrations can affect the growth as well as C6. What is more, fluopyram should be tested in combination with protiokonazol, the second active ingredient of Propulse, in order to find the lowest possible concentration for both drugs in combination as well as individually.

4.2 Soil assay

The aim of this pilot project was to assess if the type of soil and the soil condition influence *A. solani* growth and survival. The four different soils collected from potato fields had different sand content, PET was the soil with the highest sand content (19%), then VIK with 12%, followed by JAR with 5.6% and finally HAK with 3.5%. All four types of soil were divided into three conditions: sterilized, unsterilized wet and unsterilized dry. Rye kernels inoculated with *A. solani* mycelia were dug into the different soils and the amount of fungus was estimated after 66 days of incubation by comparing the weights of the kernels before and after the soil incubation and using quantitative PCR. The infected kernels responded differently to the different condition and type of soils. The weight of the bags with the inoculated kernels at the end of the experiment was only significantly different between HAK and the other fields. However, the difference in weight between the weight before and after incubation in the soil was lower for HAK than the other soils. It means that the bags lost less weight than the others. It could mean that *A. solani* had a better survival in the soil of this field, with the lowest sand content. It is important to note, however, that the increase in the pathogen's biomass, as the fungus grows leads also to a decrease in the weight of the kernels, as the fungus feeds on the rye. Therefore, it is not possible to state that the HAK bags contained more *A. solani* than the other bags based on the weight alone. A possible explanation for the weight difference is the fact that the sandy soils do not retain water as well as the clay ones because of their composition. This would mean that the kernels from the clay soils were more humid, and it could influence their weight. This could also be explained as the clay and sandy soils do not have the same

composition and nutrients content, which could results in a different way of affecting the survival of *A. solani*, this hypothesis is also approached on Stridh *et al.* (unpublished data). Stridh *et al. (*unpublished data) results suggested that the type of soil, clay and sandy, is essential for the severity of *A. solani* infection. They explained it by hypothesizing that it is easier for lighter soils (sandy) to run out of nutrients than clay soils. As well as clay soils have different microbiome than sandy ones, so it could lead to more competition for the pathogen (Stridh et al., unpublished data).

The weights of the kernels incubated in the HAK soil were significantly different from the other soil types under all tree conditions. It could mean that moisture that is retained better in the clay soil, like HAK, is important for the survival of *A. solani*. HAK is also the soil with the least potassium content and has quite high phosphorus content. Potassium effect is still discussed and depends on the organisms. Indeed some studies said that a high level of potassium decreases the disease incidence, but it is not always the case (Amtmann et al., 2008).

The qPCR analysis allowed to quantify *A. solani* much more precisely, because it was based on the *A. solani* DNA concentrations in the kernels. The different types of soil did not have a significant impact on *A. solani* DNA concentration.

From the three conditions, the dry condition was significantly different from the other two, and the one in which the differences in kernel weights before and after the soil incubation were the smallest. It suggests that the survival of *A. solani* was the highest in this condition. This was confirmed by the qPCR results, where dry condition had the highest DNA concentrations in all four soil types. This means that *A. solani* survived better under the dry condition. These results correlate well with the finding of Jindo *et al.* (2021) that stated that *A. solani* survive in the soil over different seasons particularly when the soil is dry. Also the soil moisture seems to have an impact on the incidence of differents diseases (Ghorbani et al., 2008). However it also depends on the pathogen, as not all pathogens react the same way to the moisture content (Fiers et al., 2012).

The DNA concentration for sterilized and unsterilized conditions were largely the same under the same soil type. It was relatively low, which means that *A. solani* survival is more compromised than under the dry condition. These two conditions have one thing in common - moisture. We can speculate that moisture plays a negative role in the survival of *A. solani* in the soil. However, it is important to keep in mind that only the rye kernels were used for DNA extraction and not the soil. It is possible that *A. solani* had spread in the soil, and that the concentration of *A. solani* in the soil was higher than that in the bags. It will thus be possible, depending on the results, to know whether moisture favours the spreading of *A. solani* but also to support or not our results on the survival of *A. solani.*

The weight analysis can be biased, as the mycelium is heavier than the spores. It is then possible that the bags were very light because they contained spores rather

than mycelium. It would be interesting for future studies to quantify the mycelium and spores present in the bags to optimally estimate *A. solani* survival.

It is also important to highlight the fact that the soils were not analysed after the inoculation of the kernels. *A. solani* survival was assessed only through the kernel weights, however it was noted that, particularly under the dry condition, the mycelium was visibly developing on the bags. It is then probable that *A. solani* spread to the soil. Thus, to estimate *A. solani* growth and survival in the soils, it will be needed in the future to analyse the soil.

These results can be concerning regarding the expected scenario for the global warming. It is expected that the temperature will rise, as well as the carbon dioxide levels. It also has been discussed that pathogenicity of *Alternaria spp.* can increase with higher temperatures according to carbon dioxide levels (Gullino et al., 2018). The temperature rising will result in water shortage, so if *A. solani* survives better in a dry soil, it can lead to severe epidemic and crop losses that will affect billions of people. Water shortage in the agricultural world is already a problem for developing countries, but it also touched the developed ones. Europe has a lot of countries that are regulating the usage of water during summer such as France or Italy.

Conclusion

The mycelia growth depends of the fluopyram concentration levels as well as the year the sample was collected. The previously applied treatment (fungicide or not) did not have an influence on the mycelia growth, yet a loss of sensitivity to fluopyram was observed. The soil conditions, unsterilized dry, sterilized or unsterilized wet, have respectively either a positive or no influence on the survival of *A. solani.* These results are quite concerning looking at the expected global warming and the actual agricultural land management. It is clear that more IPM methods need to be applied on the agricultural land to prevent epidemic and increase of yield losses. More research about the relationship between *A. solani* and the type of soil and its composition need to be carried out in order to better integrate the IPM methods but also to have a clearer understanding on how to approach the future years for potato crops. There is then a need to review our agricultural land use to face the future more prepared and apprehend actual and upcoming environmental challenges.

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Popular science summary

Potatoes are among the most important crops around the world, as they feed millions of people. However, potatoes are subject to diseases that can cause many yield losses and therefore can impact the food industry. One of the diseases that can affect potatoes is early blight. It is a disease which symptoms can be seen on mature leaves as blackish dry round lesions. It is caused by a fungus called *Alternaria solani*. *A. solani* is a soil-borne fungus, which means it lives in the soil. This fungus is recognisable when grown on agar plates, by its dark ring succession and its spores, which are reproductive cells that grow on the fungus mycelia. The mycelium is the filamentous part of the fungus that is uptaking nutrients from the soil. Early blight can cause a lot of damage to potato crops and can be found all around the world. It is needed to control this pathogen to prevent it to cause too much losses. One of the most employed control methods is the fungicide application. Different types of fungicides can be utilized, including the SDHI and QoI ones. The SDHI fungicides are fungicides that inhibit fungal growth by blocking an enzyme that induces cell respiration, so it reduces the cell respiration which leads to less energy being liberated. Among these fungicides, we can find a chemical called fluopyram. It is a fungicide that is used as an active component of the new fungicide of Bayer: Propulse. This fungicide is, for a few years now, used to treat early blight in potato crops. Fluopyram is a chemical that is toxic with long-lasting effects on aquatic life. This problem is shared with a lot of other fungicides and as the fungicide does not disappear once in the soil, it contaminates the water. Water contamination is an environmental problem but fungicides can also cause health problems in humans, such as asthma or cancer. The use of fungicides needs to be reassessed and other methods need to be investigated. Some alternative methods are the integrated pest management methods (IPM). The IPM methods are encouraging a more responsible use of fungicides, and integrating less aggressive methods that take into account the environment. But the IPM methods are not integrated into the land management practices enough. The overuse of fungicides may lead to the development of resistance of the pathogen to the fungicide. *A. solani* resistance development to fungicide has been reported, and may have been caused by mutations. It is then needed to look at the resistance development of *A. solani* with fluopyram in order to prevent it but also to have a full understanding on how *A. solani* is acting in the soil. Our investigation showed that the increase of

fluopyram concentration leads to decreased growth of *A. solani*, but also that the sensitivity to flupyram decreases with the years. It means that fluopyram have a weaker influence on *A. solani* growth. It may indicate that in the future years *A. solani* will become resistant or insensitive to fluopyram if its use is not adapted. The results also indicate that a lower content of fluopyram could be used in Propulse, which is important knowing the effect of fluopyram on the environment. The soil condition, such as dry soil or wet soil, have an effect on *A. solani* survival as well as if the soil has been clear of other organisms or not. *A. solani* survived better in dry soils. This discovery is quite alarming knowning that the temperature will rise, which will induce water evaporation and lead to more dryer soil. It can then be expected that *A. solani* will survive better in the future climate and may create epidemic if nothing is done about it. More studies are needed to assess the interactions between *A. solani* and the soil, to know if a specific dry level could lead to more of the fungus survival. A follow-up on these questions is required in order to prevent more damage of *A. solani* in the future. Also to try to re-evaluate the way we are currently treating *A. solani* to find an optimal way where we could take into account the environmental, human health and economic problems that are caused intrinsically by our land management methods.

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Appendix 1


Nutrient results eurofins.pdf

This pdf files contains the full analysis report performed on our soil from Eurofins. The soil we used for the soil assay are HAK,JAR, PET1 , and VIK2, referenced in our text as HAK,JAR,PET and VIK.

Appendix 2

Treatment	Concentration	Year	ID sample	Sporulation
Treated	C0	2018	N3.3.10.1	yes
Treated	C0	2018	N3.3.10.1	no
Treated	C0	2018	N3.1.10.1	yes
Treated	C0	2018	N3.1.10.1	yes
Untreated	C0	2018	N2.2.1.4	no
Untreated	C0	2018	N2.2.1.4	yes
Untreated	C0	2019	N19.1.1	yes
Untreated	C0	2019	N19.1.1	yes
Treated	C0	2019	N19.3.3B	yes
Treated	C0	2019	N19.3.3B	yes
Untreated	C0	2020	N20.1.3	yes
Untreated	C0	2020	N20.1.3	yes
Untreated	C0	2021	N21.5.3	yes
Untreated	C0	2021	N21.5.3	yes
Treated	C0	2021	N21.7.1	no
Treated	C0	2021	N21.7.1	yes
Treated	C0	2021	N21.7.3	yes
Treated	C0	2021	N21.7.3	yes
Treated	C0	2022	L22.6.1	yes
Treated	C0	2022	L22.6.1	yes
Treated	C0	2022	L22.6.3	yes
Treated	C0	2022	L22.6.3	yes
Untreated	C0	2022	L22.3.2	yes
Untreated	C0	2022	L22.3.2	yes
Untreated	C0	2022	L22.3.3	yes
Untreated	C0	2022	L22.3.3	yes
Treated	C1	2018	N3.3.10.1	yes
Treated	C1	2018	N3.3.10.1	yes
Treated	C1	2018	N3.1.10.1	no
Treated	C1	2018	N3.1.10.1	no
Untreated	C1	2018	N2.2.1.4	no
Untreated	C1	2018	N2.2.1.4	no
Untreated	C1	2019	N19.1.1	yes
Untreated	C1	2019	N19.1.1	yes
Treated	C1	2019	N19.3.3B	yes
Treated	C1	2019	N19.3.3B	yes

Untreated	C1	2020	N20.1.3	yes
Untreated	C1	2020	N20.1.3	yes
Untreated	C1	2021	N21.5.3	yes
Untreated	C1	2021	N21.5.3	yes
Treated	C1	2021	N21.7.1	yes
Treated	C1	2021	N21.7.1	yes
Treated	C1	2021	N21.7.3	yes
Treated	C1	2021	N21.7.3	yes
Treated	C1	2022	L22.6.1	yes
Treated	C1	2022	L22.6.1	yes
Treated	C1	2022	L22.6.3	yes
Treated	C1	2022	L22.6.3	yes
Untreated	C1	2022	L22.3.2	yes
Untreated	C1	2022	L22.3.2	yes
Untreated	C1	2022	L22.3.3	yes
Untreated	C1	2022	L22.3.3	yes
Treated	C2	2018	N3.3.10.1	no
Treated	C2	2018	N3.3.10.1	yes
Treated	C2	2018	N3.1.10.1	yes
Treated	C2	2018	N3.1.10.1	yes
Untreated	C2	2018	N2.2.1.4	yes
Untreated	C2	2018	N2.2.1.4	yes
Untreated	C2	2019	N19.1.1	yes
Untreated	C2	2019	N19.1.1	yes
Treated	C2	2019	N19.3.3B	yes
Treated	C2	2019	N19.3.3B	yes
Untreated	C2	2020	N20.1.3	yes
Untreated	C2	2020	N20.1.3	yes
Untreated	C2	2021	N21.5.3	yes
Untreated	C2	2021	N21.5.3	yes
Treated	C2	2021	N21.7.1	yes
Treated	C2	2021	N21.7.1	yes
Treated	C2	2021	N21.7.3	yes
Treated	C2	2021	N21.7.3	yes
Treated	C2	2022	L22.6.1	yes
Treated	C2	2022	L22.6.1	yes
Treated	C2	2022	L22.6.3	yes
Treated	C2	2022	L22.6.3	yes
Untreated	C2	2022	L22.3.2	yes
Untreated	C2	2022	L22.3.2	yes
Untreated	C2	2022	L22.3.3	yes
Untreated	C2	2022	L22.3.3	yes
Treated	C3	2018	N3.3.10.1	yes
Treated	C3	2018	N3.3.10.1	yes
Treated	C3	2018	N3.1.10.1	yes
Treated	C3	2018	N3.1.10.1	yes

Untreated	C3	2018	N2.2.1.4	yes
Untreated	C3	2018	N2.2.1.4	yes
Untreated	C3	2019	N19.1.1	yes
Untreated	C3	2019	N19.1.1	yes
Treated	C3	2019	N19.3.3B	yes
Treated	C3	2019	N19.3.3B	yes
Untreated	C3	2020	N20.1.3	yes
Untreated	C3	2020	N20.1.3	yes
Untreated	C3	2021	N21.5.3	yes
Untreated	C3	2021	N21.5.3	yes
Treated	C3	2021	N21.7.1	yes
Treated	C3	2021	N21.7.1	yes
Treated	C3	2021	N21.7.3	yes
Treated	C3	2021	N21.7.3	yes
Treated	C3	2022	L22.6.1	yes
Treated	C3	2022	L22.6.1	yes
Treated	C3	2022	L22.6.3	yes
Treated	C3	2022	L22.6.3	yes
Untreated	C3	2022	L22.3.2	yes
Untreated	C3	2022	L22.3.2	yes
Untreated	C3	2022	L22.3.3	yes
Untreated	C3	2022	L22.3.3	yes
Treated	C4	2018	N3.3.10.1	no
Treated	C4	2018	N3.3.10.1	yes
Treated	C4	2018	N3.1.10.1	yes
Treated	C4	2018	N3.1.10.1	yes
Untreated	C4	2018	N2.2.1.4	no
Untreated	C4	2018	N2.2.1.4	no
Untreated	C4	2019	N19.1.1	yes
Untreated	C4	2019	N19.1.1	yes
Treated	C4	2019	N19.3.3B	yes
Treated	C4	2019	N19.3.3B	yes
Untreated	C4	2020	N20.1.3	yes
Untreated	C4	2020	N20.1.3	no
Untreated	C4	2021	N21.5.3	no
Untreated	C4	2021	N21.5.3	yes
Treated	C4	2021	N21.7.1	yes
Treated	C4	2021	N21.7.1	yes
Treated	C4	2021	N21.7.3	yes
Treated	C4	2021	N21.7.3	yes
Treated	C4	2022	L22.6.1	yes
Treated	C4	2022	L22.6.1	yes
Treated	C4	2022	L22.6.3	yes
Treated	C4	2022	L22.6.3	yes
Untreated	C4	2022	L22.3.2	yes
Untreated	C4	2022	L22.3.2	yes

Untreated	C4	2022	L22.3.3	yes
Untreated	C4	2022	L22.3.3	yes
Treated	C5	2018	N3.3.10.1	yes
Treated	C5	2018	N3.3.10.1	yes
Treated	C5	2018	N3.1.10.1	no
Treated	C5	2018	N3.1.10.1	no
Untreated	C5	2018	N2.2.1.4	no
Untreated	C5	2018	N2.2.1.4	no
Untreated	C5	2019	N19.1.1	yes
Untreated	C5	2019	N19.1.1	yes
Treated	C5	2019	N19.3.3B	yes
Treated	C5	2019	N19.3.3B	yes
Untreated	C5	2020	N20.1.3	yes
Untreated	C5	2020	N20.1.3	yes
Untreated	C5	2021	N21.5.3	no
Untreated	C5	2021	N21.5.3	no
Treated	C5	2021	N21.7.1	yes
Treated	C5	2021	N21.7.1	yes
Treated	C5	2021	N21.7.3	yes
Treated	C5	2021	N21.7.3	yes
Treated	C5	2022	L22.6.1	yes
Treated	C5	2022	L22.6.1	yes
Treated	C5	2022	L22.6.3	yes
Treated	C5	2022	L22.6.3	yes
Untreated	C5	2022	L22.3.2	yes
Untreated	C5	2022	L22.3.2	yes
Untreated	C5	2022	L22.3.3	yes
Untreated	C5	2022	L22.3.3	yes
Treated	C6	2018	N3.3.10.1	no
Treated	C6	2018	N3.3.10.1	yes
Treated	C6	2018	N3.1.10.1	no
Treated	C6	2018	N3.1.10.1	no
Untreated	C6	2018	N2.2.1.4	no
Untreated	C6	2018	N2.2.1.4	no
Untreated	C6	2019	N19.1.1	yes
Untreated	C6	2019	N19.1.1	yes
Treated	C6	2019	N19.3.3B	yes
Treated	C6	2019	N19.3.3B	yes
Untreated	C6	2020	N20.1.3	yes
Untreated	C6	2020	N20.1.3	yes
Untreated	C6	2021	N21.5.3	no
Untreated	C6	2021	N21.5.3	no
Treated	C6	2021	N21.7.1	yes
Treated	C6	2021	N21.7.1	yes
Treated	C6	2021	N21.7.3	yes
Treated	C6	2021	N21.7.3	yes

Treated	C6	2022	L22.6.1	yes
Treated	C6	2022	L22.6.1	yes
Treated	C6	2022	L22.6.3	yes
Treated	C6	2022	L22.6.3	yes
Untreated	C6	2022	L22.3.2	yes
Untreated	C6	2022	L22.3.2	yes
Untreated	C6	2022	L22.3.3	yes
Untreated	C6	2022	L22.3.3	yes

Annexe 2. Sporulation of the 14 isolates of the solid assays for all concentrations (C0-C6)

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