Fungicide resistance and soil survival of Alternaria solani

Fungicidresistens och överlevnad i jorden hos Alternaria solani

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Abstract

Alternaria solani is a major pathogen affecting potato (*Solanum tuberosum*), causing early blight and significant yield annual losses globally. Due to the pathogen's ability to develop resistance to QoI fungicides and boscalid, a SDHI fungicide, concerns have arisen regarding future fluopyram resistance. Fluopyram is a SDHI fungicide that inhibits fungal respiration and is one of the active ingredients of a relatively new fungicide, Propulse. This study aimed to assess the level of resistance of *A. solani* isolates to fluopyram and whether the isolates studied showed signs of fungicide resistance 2 years after introduction of Propulse. Another objective was to study the survival of the pathogen in soil during winter as this could influence its epidemiology. The study was separated into three experiments: a germination assay testing spore viability across fungicide concentration, the prevalence of *A. solani* on treated or non-treated leaves and an evaluation of soil type on fungus survival. The germination assay revealed a significant reduction in spore germination at high fluopyram concentrations, although it did not completely inhibit spore viability. *A. solani* was detected in all leaf samples, with fungicide-treated leaves unexpectedly containing higher levels of *Alternaria spp.*. The presence of *A. solani* despite treatment, as seen on leaf samples and germination assay, might indicate the possibility of lower sensitivity to the fungicide. The results from the soil experiment aligned with current literature, showing enhanced survival of *A. solani* in dry clay soils. These findings, although demonstrating fluopyram's current effectivity on the pathogen, hint towards future challenges. Increased temperatures tend to lead to drier soils which could promote *A. solani* infections. Although, further research is required to validate these observations.

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List of abbreviations

SLU - Swedish university of Agricultural Sciences

A. solani - *Alternaria solani*

SDHI - Succinate Dehydrogenase Inhibitor

QoI - Quinone-outside Inhibitor

CTC - Chlortetracycline

PDB - Potato Dextrose Broth

PDA - Potato Dextrose Agar

SD - Standard Deviation

SCA - Standard Curve A

SCB - Standard Curve B

SCC - Standard Curve C

SCD - Standard Curve D

SCE - Standard Curve E

SCF - Standard Curve F

Introduction

Alternaria solani

Alternaria solani is a plant-pathogenic fungus that causes early blight in the *Solanaceae* family, in which potato *(Solanum tuberosum)* belongs to. It is one of the major diseases affecting commercially grown potatoes worldwide, primarily targeting older leaves. This causes premature defoliation and thus reduced tuber yield. *A. solani* can cause yield losses up to 70-80% if left untreated (Shtienberg, et al. 1989), however in combination with fungicide and culture management strategies such as removal of infected tissue, it is rare for that number to exceed 20% (Wharton, Kirk. 2007).

Symptoms

A. solani primarily affects older leaves and manifests as small brown lesions with a characteristic "bullseye" shape. As the infection progresses and the lesions spread, the leaf ultimately becomes necrotic, reducing the photosynthetic capacity of the plant. This can further lead to inhibited tuber growth. Infection can spread to tubers as well, where symptoms manifest as sunken, dark lesions and dry skin on the tuber (Wharton, Kirk. 2007). This results is not only quantitative loss for farmers, but qualitative as well.

Environmental factors

Temperature and humidity are important factors contributing to epidemiology and development. Weather conditions fluctuating between dry and humid, with temperatures ranging between 20-30℃ seem to be favourable for spore production and inoculation of *A. solani* (Jindo et al. 2021). According to Jindo et al. (2021), in terms of disease control, the use of fungicides and crop management practices such as removal of weeds and contaminated debris and pruning can limit the risk of fungus survival. In terms of management, crop rotation has also shown to be crucial for preventing *A. solani* infection since the fungus can overwinter or survive in soil for up to 8 months (Jindo et al. 2021).

According to the review conducted by Jindo et al. (2021), annual fungicide costs in the US range between $21.4 - $44.8 million. Better understanding of when to apply fungicides, and the environmental and biological effects they have, may help reduce unnecessary use as well as associated economic costs.

Fluopyram

Fluopyram is one of the two components of the fungicide Propulse, together with prothioconazole (Bayer Crop Science, n.d). Fluopyram is a succinate dehydrogenase inhibitor (SDHI) which targets the succinate dehydrogenase enzyme, also known as Complex II of the mitochondrial electron transport chain. This class of fungicides inhibits cell respiration. In comparison, another major fungicide group are Quinone-outside inhibitors (QoI) which target

complex III in the electron transport chain (Gudmestad et al. 2013). Due to the difference in how the fungicides target respiration, both groups tend to be used in tandem with each other to maximize effectivity.

Development of resistance

QoI fungicides have been widely used in the management of *A. solani* but the appearance of mutations in *A. solani* species, for example F129L or G143A, have been associated with reduced sensitivity in isolates. These mutated variants showed an increased tolerance to QoI fungicides and they also rapidly developed resistance towards boscalid, another SDHI-classified fungicide (Gudmestad et al. 2013). Therefore, concerns of dual fungicide resistance arose. However, the isolates with the F129L mutation showed no increased resistance towards fluopyram.

A study conducted by Gudmestad et al. (2013) tested the boscalid resistance in *A. solani* and what resistance for boscalid means for other SDHI fungicides, including fluopyram. The study showed the isolates that were resistant to boscalid remained sensitive to fluopyram. Another study conducted by Einspanier et al. (2022) analysed 48 *A. solani* isolates from across Europe and found that SDHI resistance developed independently. Therefore, the study concluded that resistance did not evolve due to one resistant isolate, but rather the species evolved multiple times to withstand the use of SDHI fungicides. However, the mechanisms behind these findings have not been established and due to the species' ability to develop resistance independently, concerns regarding future fluopyram tolerance remains.

Research questions

The aim of this study is therefore:
1. To determine whether fluopyram, a component of the fungicide Propulse, effectively inhibits spore germination in *A. solani,* with the hypothesis that higher concentrations reduce spore germination rates.
2. To assess whether *A. solani* can be isolated from treated and untreated potato leaves to evaluate the persistence of the pathogen two years after fungicide application, with the hypothesis that fungicide treated samples will contain less *Alternaria* species.
3. To investigate how different soil types and moisture conditions affect fungus survival in the soil over winter, with the hypothesis that drier soils promote survival of the pathogen.

Materials and methods

A. solani media cultures

Throughout the experiments, *A. solani* was maintained on a variety of media. The water agar was made by dissolving 7.5 g of bacto agar in 500 mL of MilliQ water and autoclaved at 121

C for 21 min. Plates with 20% Potato Dextrose Agar (PDA) were prepared by dissolving 3.9 g of potato dextrose agar and 6 g of bacto agar in 500 mL of MilliQ water, autoclaved at 121 C for 20 min and stored in sealed bags. The Potato Dextrose Broth (PDB) was made by dissolving 2.4 g of potato dextrose broth in 100 mL of MilliQ water and autoclaved at 121 C for 21 min. PDA and water agar plates were supplemented with 25 ug/mL of the antibiotic Chlortetracycline (CTC) when required by the experiment.

Germination experiment

The germination experiment was done to test for potential reduced sensitivity across different concentrations of fluopyram, one of the two components of the commonly used Propulse fungicide. The isolates were collected in 2023 from infected potato fields.

Sample preparation

Five *A. solani* isolates were cultured by placing a 1 x 1 cm of agar plug infected with *A. solani* on a 20% PDA plates and incubated in the dark for 7 days to promote mycelial growth

Afterwards, they were transferred to the UV-incubator for 7-8 days to promote sporulation. After 7-8 days, the spores were dislodged by dropping 10 ml of distilled water onto the plate and by gently scraping for up to a minute using a L-shaped glass cell spreader. The L-shaped spreader was cleaned with ethanol and bleach between isolates. The spores suspended in water was transferred to a Falcon tube. Fifteen µl from the spore suspension was transferred to a Fuchs-Rosenthal counting chamber, and the spores counted under a microscope. The resulting number of spores was divided by 0.0032 to determine the spore concentration/mL. For each isolates, the spores were counted twice, and then the mean was calculated. To standardize the spore quantities between isolates, each isolate was either diluted or concentrated to a common concentration of 2 x 10^4 spores/mL.

For the isolates that needed concentrating, the Falcon tube was centrifuged and emptied of the water using a pipette. The amount of water added to the Falcon tube was calculated using $C_1 V_1 = C_2 V_2 \rightarrow V_2 = \frac{(C_1 \cdot 10)}{20000}$ where the starting volume, V_1, was 10 µl.

For the isolates that needed diluting, the amount of water needed to be added was calculated similarly, using $C_1 V_1 = C_2 V_2 \rightarrow V_2 = \frac{(C_1 \cdot 10)}{20000}$. However, the starting volume, V_1, was determined to be 1 mL since the spore count is decided per mL.

Germination assay

For the germination assay, water agar plates were made and amended with the concentrations of fluopyram of Table 1. The plates were referred to as C_0, C_1, C_2, C_3, C_4, C_5 and C_6 for their increasing levels of the fungicide concentration, with C_0 being a water agar plate with no fungicide and C_6 being the plate with the highest fungicide concentration.

Table 1: Table showing the volume of fluopyram added to the 500 mL of medium bottle and the relative concentrations of each plate.

Plate code	Volume of fluopyram added to 500 mL water agar bottle	Relative concentration (µg/mL)
C_0	0	0
C_1	5 µl	0.01
C_2	50 µl	0.1
C_3	500 µl	1
C_4	5 ml	10
C_5	25 ml	50
C_6	50 ml	100

Once the plates were ready, 50 µl of the spore suspension from each isolate was pipetted to two replicate plates for each concentration and evenly spread by using an L-shaped glass cell spreader. Thereafter, the plates were sealed with parafilm and incubated in the UV chamber for 5 hours. After incubation, the plates were examined under a stereoscope, and the number of germinated and non-germinated spores were counted up to a total of 100 in order to calculate the germination percentage. A spore was considered germinated when the sporulation tube was at least half as long as the spore itself.

Leaf isolation

The aim for this experiment, as mentioned previously, is to primarily detect and quantify *A.solani* and other *Alternaria* species (e.g., *A. alternata*) commonly co-infecting potato leaves. Species recognition was done based on spore morphology. And second, to establish single-cell cultures of *A. solani* for future fungicide assays. Infected potato leaves were collected from field trials in Mosslunda (Kristianstad Municipality) in early September 2024 from the following treatments: untreated plots, treated with either 100% Propulse or a alternating application of Propulse, Revyona, Narita and Revyona again, referred to as Lyckeby strategy in this study.

Step 1

The leaves were examined to detect the bulls-eye spots, typical of *A. solani* infection. One lesion was chosen per leaf. A segment of the infected leaf containing half of the lesion and half of healthy tissue was cut with a scalpel and washed in 70% ethanol, 10% bleach solution, and rinsed with MilliQ water. Thereafter, it was placed on a water agar plate supplemented with 25 g/ml of the antibiotic CTC. For each plate, six leaves per treatment were selected and one piece taken from each leaf. The plates were sealed with parafilm and transferred to a UV incubator at 18℃ and 30% near-UV light from 8 am to 5 pm for 7 days to promote mycelium growth and sporulation . The plates were named according to the year the samples were taken, the treatment received (1 for untreated, 2 for treated), the block and the leaf number which were noted on Excel. A visual reference of one of the plates is provided in Figure 1.

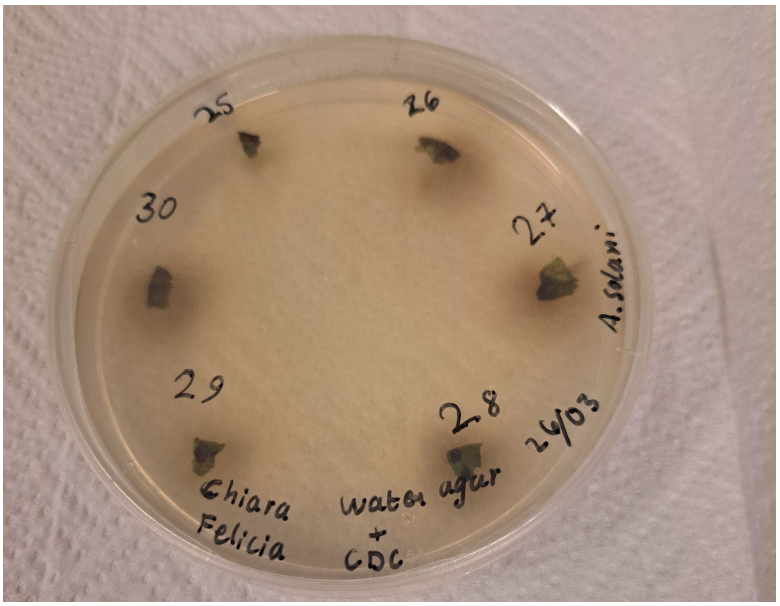

Figure 1: Picture showing a plate with leaf segments. Note that CTC has accidentally been marked as CDC.

Step 2

After 7 days, the plates were examined under a stereoscope. For each leaf piece, the percentage of *A. solani* and other *Alternaria* species were noted. Once *A. solani* spores were identified, their position on the plate was marked. Under a sterile hood, the spores were collected by pressing a plastic loop against the medium and gently scraped. Once the spores had been collected, they were transferred to a 20% PDA plate. Each replicate was placed on an individual petri dish, sealed with parafilm and placed in the UV incubator for an additional 7 days.

Step 3

After 7 days, the plates were examined under a stereoscope and the position of *A. solani* spores was marked and dislodged by pipetting 40 µl MilliQ water up and down into the spot repeatedly. Once the spores were detached, the suspension was transferred onto a new 20%

PDA plate and spread with an L-shaped glass spatula to spread out the spores. The plates were then incubated in the UV chamber for 3 hours to facilitate spore germination. The germinated spores were identified based on the signs of hyphal emergence. The exact position of the spore was marked and a scalpel was used to cut a piece of the agar with the spore and transfer it to a new 20% PDA plate, resulting in the creation of a single-cell isolate. This plate was sealed and incubated in the UV incubator for 8 days.

Validation of *A. solani* isolation

Once the single-cell cultures of *A. solani* were obtained, they had to be confirmed through qPCR. A 1 cm x 1 cm piece of agar was cut out from the outer edge of the mycelium and placed inside a PDB bottle. These bottles were then stored in the dark at room temperature for 7 days.

DNA Extraction

The mycelium that grew in the PDB bottle was harvested by draining the liquid through a 40 µL mesh filter and dabbed on paper to drain the excess liquid. The mycelium was separated from the agar plug and placed inside of a mortar with liquid nitrogen. While frozen, the mycelium was ground into a powder and transferred into a tissue disruptor tube from the DNeasy Pro Plant Kit from Qiagen.

The DNA was extracted from the frozen powder by following the protocol provided by the manufacturer (Qiagen kit). Briefly, 500 uL of CD1 lysis solution was added to each disruption tube and vortexed for 5 minutes to homogenize the samples. The tubes were then centrifuged at 12,000 rpm for 2 minutes. The supernatants were transferred to microcentrifuge tubes and 200 mL of CD2 solution was added and vortexed for 5 s. The tubes were then centrifuged at 12,000 rpm for 1 min. The supernatants were transferred to clean microcentrifuge tubes and 500 µL of APP buffer was added. The tubes were vortexed for 5 s, after which 600 µL of the lysate was loaded onto an MB Spin Column and centrifuged at 12,000 rpm for 1 min. The MB spin column was placed into a clean collection tube and 650 µL of Buffer AW1 was added and centrifuged at 12,000 rpm for 1 min . The flow-through was discarded and 650 µL of Buffer AW2 was added and centrifuged as above. The flow-through was discarded and the tubes were centrifuged again at 13,200 rpm for 2 minutes. The MB spin columns were placed into elution tubes and 50 µL of Buffer EB was added to the centre of the filter and centrifuged at 12,000 rpm for 1 minute. The MB columns were then discarded. This last step yielded to 50 uL of DNA solution for downstream analyses.

After the extraction, the DNA in the samples were quantified via the NanoDrop spectrometer.

qPCR protocol

For the qPCR, the lab strain As112 was used as a positive control and sterile water was used as a negative control. The qPCR reaction was performed with CFX96 thermal thermocycler (Bio-Rad) using 10 µL 2x SYBR green qPCR Master Mix (Thermo Fisher Scientific), 0.4 µL

of both forward (5'-CCA CCA GTG ATC CGG GAATA-3') and reverse (5'-GTGGGAGGGGTCACATAACT-3') primer (10 µM), 8.2 µL nuclease free water and 1 µL of the template DNA, for a total volume of 20 µL. The reaction was performed at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 64 °C for 10 s, extension at 72 °C for 10 s. The fluorescence was read at 72 °C at the end of each cycle, followed by the analysis of the melting curve at 65 – 95 °C with an increment of 0.5 °C. Each sample was run in triplicates.

Soil experiment

Kernel inoculation and soil assay

To test for the effect of soil texture (i.e., sandy vs clay soils) and drought on the long-term survival of *A. solani* in the soil, rye kernels inoculated with *A. solani* were dug down in pots in a semi-controlled setup on Alnarp campus for a period of ca. 4 months (November 2023 to February 2024). The kernel inoculation was performed by following the protocol established by Adolf and Hausladen (2015) with some adjustments. Briefly, rye kernels were autoclaved twice at 121 C for 20 min by adding 80 mL of distilled water to the bag every 150 g of kernels. Once autoclaved, the kernels were inoculated with *A. solani* lab strain (As112) by manually crushing half a plate of a solid culture every 150 g of kernels. The kernels were then incubated at 25 C for 14 days. After the two weeks, 150 g of infected kernels were placed into organza bags and dug into 3 L pots containing soil that was collected from 10 agricultural fields within Kristianstad Municipality. All fields had potato in the crop rotation, usually every fourth year. Part of the infected kernels were frozen after the 14 days, quantified through qPCR, and used as reference quantity to compare with the amount of *A. solani* found in the retrieved bags. The pots were then divided in two treatments; half of the pots experienced drought (i.e., they were covered to only allow 30% of rainfall to reach the soil) while the other half experienced regular exposure to rain. In total, 100 pots/bags were tested at the beginning of the experiment but only 51 bags were retrieved as the remaining were destroyed by animals in the field.

Once the bags were retrieved, the DNA was extracted from the kernels using the DNeasy Pro Plant Kit from Qiagen and following the manufacturer protocol. The quality and quantity of the DNA extraction was checked with Nanodrop.

qPCR on DNA extracted from kernels

For my thesis, I continued the experiment by diluting the DNA samples to a concentration of 50 ng/µL and by performing the qPCR to quantify the amount of *A. solani* that survived.

DNA sample dilution

In order to dilute the DNA, I used the formula $C_1 V_1 = C_2 V_2$, where C1 was the starting concentration of the DNA sample calculated with the Nanodrop, C2 was the final concentration of 50 ng/µL, V2 the final volume (in 10 µL of MilliQ water). The calculated DNA volume (V_1) was transferred to a new Eppendorf tube and .

Preparation of serial dilution

Before the qPCR, a serial dilution was prepared. The purpose of the serial was to construct the standard curve to quantify the DNA extracted from the kernels. The serial dilution was made using the DNA extracted from *A. solani* lab strain (As112) and by using the $C_1 V_1 = C_2 V_2$ formula. The starting concentration (C_1) was 510 ng/µl and the final concentration (C_2) was chosen to be 50 ng/µl in the final volume (V2) of 15 µl. The volume of DNA to transfer to a new Eppendorf tube (V_1) was calculated and thereafter the resulting number was subtracted from 15 in order to give the volume of MilliQ water to add. This was done to prepare the first tube with the highest concentration of DNA which was designated SCA (Standard Curve A) as shown in the table below.

Thereafter, 1 µl of SCA was transferred into tube SCB (Standard Curve B) with 9 µl of MilliQ water which was vortexed. 1 µl of the SCB solution was transferred into tube SCC and so on until SCF was prepared. This resulted in a serial dilution with the following DNA concentrations in the table:

Table 2: Table showing the serial dilutions and their DNA concentrations.

Serial dilution	DNA concentration
SCA	50 ng/µl
SCB	5 ng/µl
SCC	0.5 ng/µl
SCD	0.05 ng/µl
SCE	0.005 ng/µl
SCF	0.0005 ng/µl

qPCR protocol

The qPCR reaction was performed as described in the “Leaf isolation” experiment. Each DNA sample was run in triplicate including the serial dilution on each plate.

Results

Germination assay

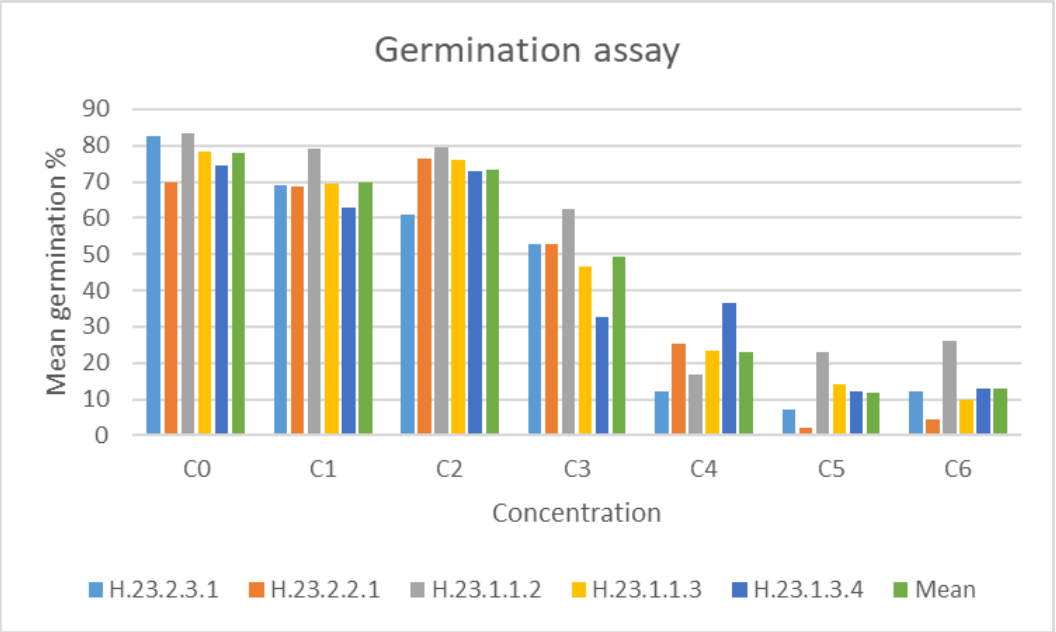

Figure 2: Bar plot depicting the results of the germination assay. The mean germination percentage on the y-axis and the concentration on the x-axis.

The results show bars representing each isolate tested, with an additional bar representing the mean result of all isolates per concentration. A decreasing trend is observed as fluopyram concentration increases. Many isolates showed an increase in spore germination percentage between C_1 and C_2, despite the overall trend of germination decreasing with higher fluopyram concentrations.

E50 refers to the concentration at which 50% of the growth is inhibited by the fungicide. Although this was not formally calculated, the mean germination percentage dropped below 50% at concentration C_3, suggesting E50 lies within this concentration.

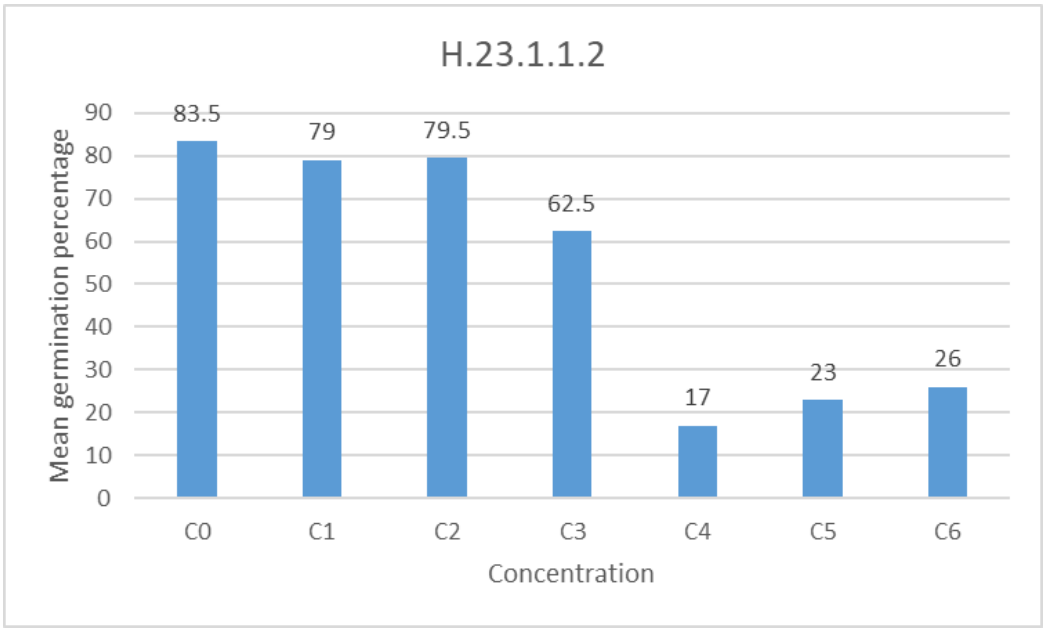

Figure 3: Bar graph depicting the results of the germination assay for isolate H.23.1.1.2.

The results are illustrated using bar plots to represent the germination assay for this specific isolate. For concentration C_4, one duplicate plate was excluded due to insufficient spore count and therefore the germination percentage was calculated based on one single plate.

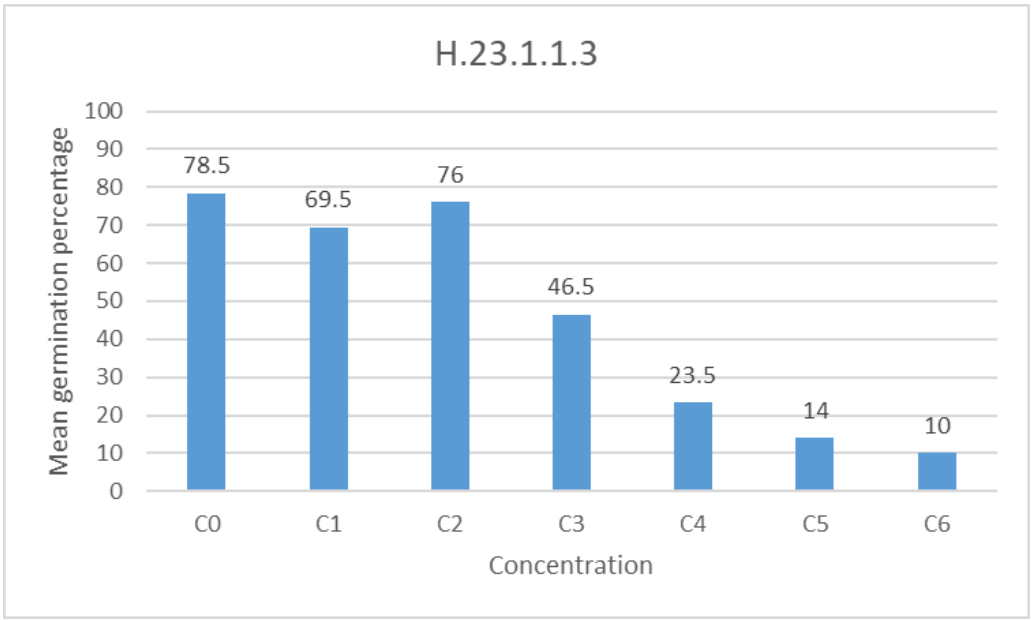

Figure 4: Bar graph depicting the results of the germination assay for isolate H.23.1.1.3.

The results are presented as bar plots representing the germination assay for this specific isolate. The results for isolate H.23.1.1.3 clearly show a consistent decreasing trend, although with an increased germination percentage between C_1 and C_2.

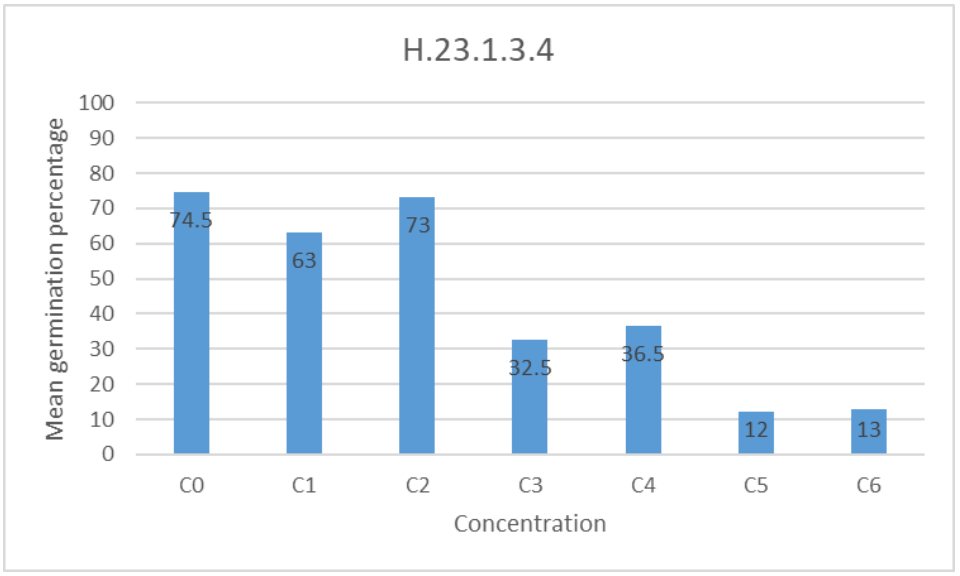

Figure 5: Bar graph depicting the results of the germination assay for isolate H.23.1.3.4.

The results are presented as bar plots representing the germination assay for this specific isolate. The results shows a clear trend of mean germination percentage decreasing over concentration. To note for this isolate is that one replicate each for concentrations C_2 and C_5 were excluded due to insufficient spore count and therefore the germination percentage for both concentrations were calculated using one of the duplicates.

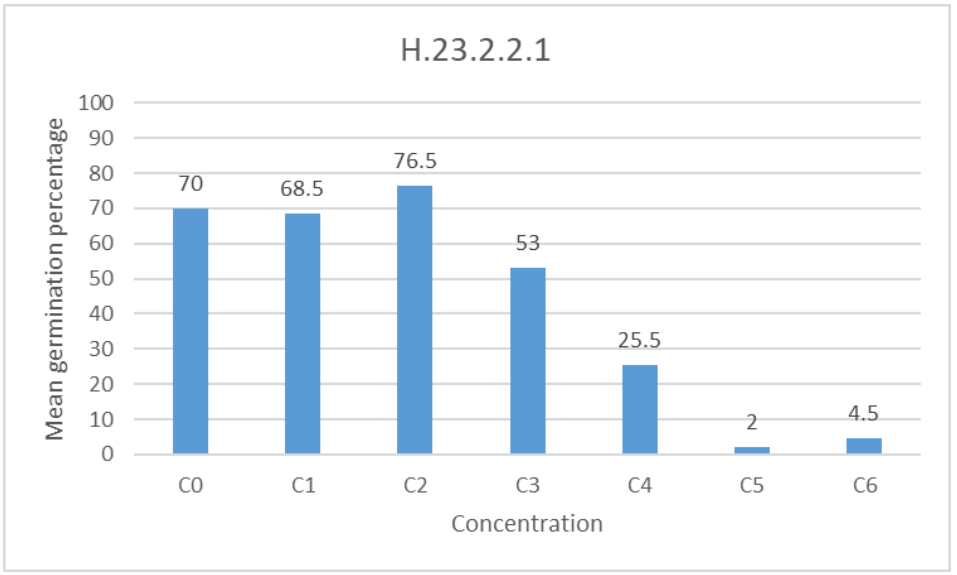

Figure 6: Bar graph depicting the results of the germination assay for isolate H.23.2.2.1.

The results are presented as bar plots representing the germination assay for this specific isolate. The results shows a clear trend of mean germination percentage decreasing over concentration. To note is that one replicate for concentration C_0 was excluded due to insufficient spore count and therefore the germination percentage was calculated using one single plate.

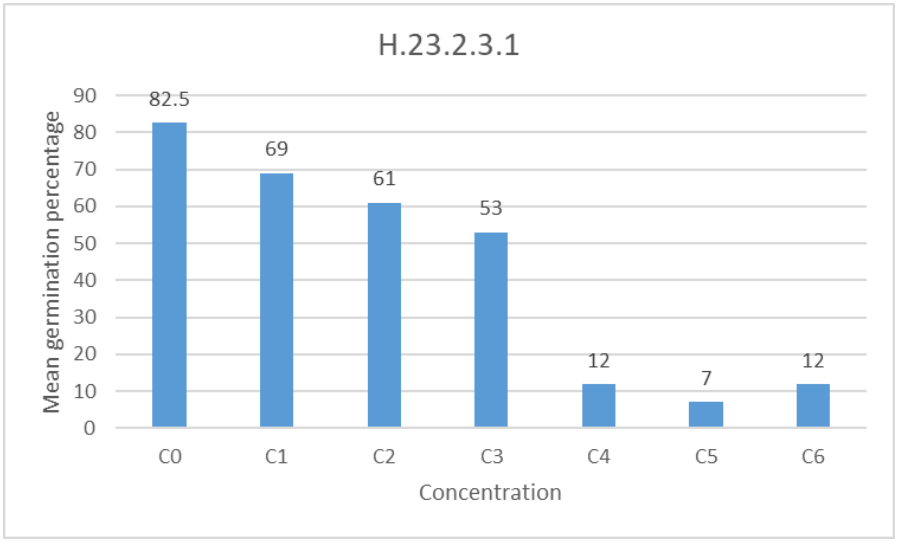

Figure 7: Bar graph depicting the results of the germination assay for isolate H.23.2.3.1. The results shows a clear trend of mean germination percentage decreasing over concentration.

The results are presented as bar plots representing the germination assay for this specific isolate. The results shows a clear trend of mean germination percentage decreasing over concentration. To note is that one replicate each for concentrations C_2 and C_5 were excluded due to insufficient spore count and therefore the germination percentage for both concentrations were calculated using one of the duplicates.

Leaf isolation

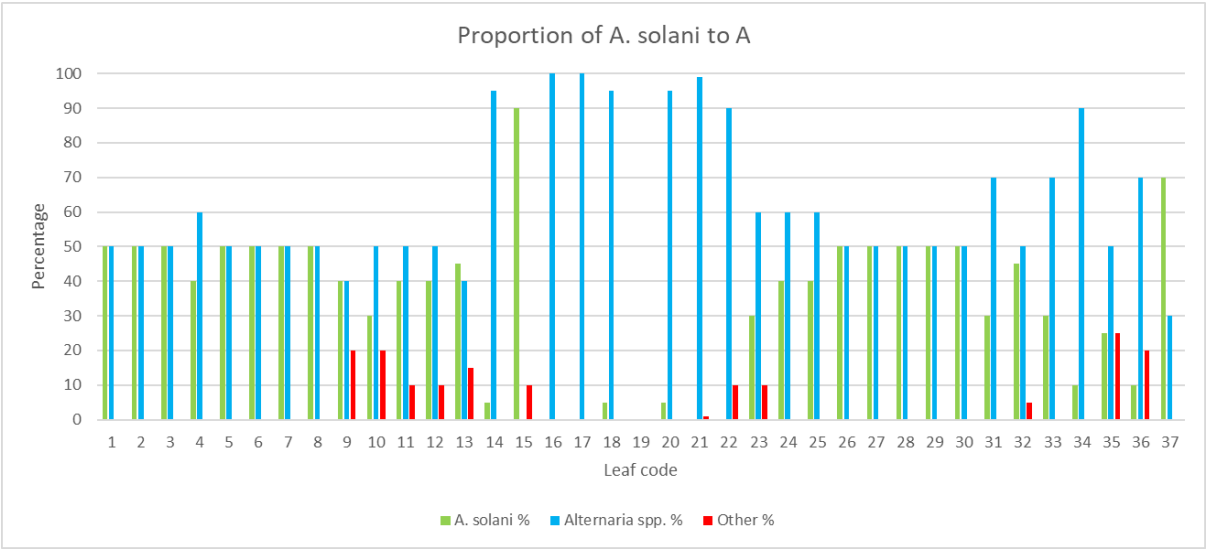

Figure 8: Graph depicting the percentage of A. solani compared to Alternaria spp. and unclassified organisms. Number 1-12 were untreated, number 13-24 were treated with propulse and number 25-37 treated with the Lyckeby strategy.

The graph above shows the results of the leaf isolation experiment. Leaves 1-12 were untreated, 13-24 were treated with fluopyram and 25-37 treated with the Lyckeby strategy. The lowest observed proportion of *A. solani* for the untreated leaves were 30% and most samples showed approximately an equal amount of *A. solani* and *Alternaria spp.,* with samples 9-12 showing presence of other unidentified organisms. The samples treated with fluopyram showed a notable presence of *Alternaria spp.*, with the group occupying up to 100% of the observed organisms and the lowest recorded proportion being 60%.

Due to the presence of unidentified organisms that, in certain samples, occupied a notable portion of the plate, it was decided to add "Other" as a category to the data.

To provide an overview of the distribution across treatments, the mean of each treatment was calculated. For the untreated samples, number 1-12, the mean proportion of *A. solani* was 45%. For the samples treated with Propulse, number 13-24, the mean proportion of *A. solani* was 19.5%. The samples treated with the Lyckeby strategy, number 25-37, showed a mean proportion of *A. solani* of 39.2%%.

The samples to be analyzed were chosen after whether they were untreated or treated, however three samples from the Lyckeby strategy were chosen (25, 27, and 29) for comparison purposes. To confirm that the 18 isolates we decided to analyze were *A. solani,* the DNA was extracted from the mycelium and performed qPCR analysis. The quality and quantity of the DNA extracted was assessed with Nanodrop. The quality of the samples is measured through the 260/280 ratio and 260/230 ratio and it should fall within a range of 1.8-2. Values that fall outside of the desired range indicate a potential contamination with either organic solvent (first ratio) or with protein/salt (second ratio). The quantity of the DNA is measured in ng/µL.

Table 3: *Results from the nanodrop for the leaf isolation.*

Leaf code	DNA_conc(ng/uL)	260/280	260/230	Date_qPCR
1	104.1	1.9	2.1	28.4.2025
2	97.6	1.9	1.8	28.4.2025
3	76.5	1.9	1.2	28.4.2025
4	89.4	1.9	1.9	28.4.2025
6.1	10.8	1.3	0.5	28.4.2025
6.2	46.7	1.8	2.1	28.4.2025

8	55.4	1.9	1.9	28.4.2025
9	43.3	1.9	1.8	28.4.2025
10	93.9	1.9	1.3	28.4.2025
13	50.7	1.9	1.7	28.4.2025
14	65.8	1.9	2.1	28.4.2025
17	90.7	1.8	0.4	28.4.2025
21	76.0	1.9	2.0	28.4.2025
22	103.6	1.9	2.1	28.4.2025
23	95.9	1.8	2.1	28.4.2025
25	73.6	1.9	1.9	28.4.2025
27	88.3	1.9	1.4	28.4.2025
29	117.6	1.9	2.1	28.4.2025

A few samples fall outside the desired range (1.8-2.0). 1, 6.2, 14, 22, 23, and 29 all showed a value above 2.0 and 3, 6.1, 10, 13, 17 and 27 showed values below 1.8. This indicates salt or protein contamination in the samples.

The qPCR results confirmed that all the isolates were *A. solani,* as shown in the table below. The negative control (H_20) was not amplified, ensuring that the reagents used for the qPCR reaction were not previously contaminated with *A. solani*.

Table 4: Table showing sample code with corresponding Ct mean value and standard deviation.

Sample number	Ct mean value	SD
As112	19.48	2.40
H_2O	NaN	NaN
1	17.08	0.15
2	18.11	1.10
3	19.36	1.99
4	16.67	0.62
6.1	16.00	0.39

6.2	18.92	1.29
8	17.77	1.34
9	17.25	0.41
10	16.41	0.09
13	17.28	0.91
14	17.31	1.28
17	15.86	0.59
21	16.33	0.28
22	16.72	1.11
23	16.71	0.98
25	16.34	0.60
27	16.20	0.32
29	16.09	0.89

Soil experiment

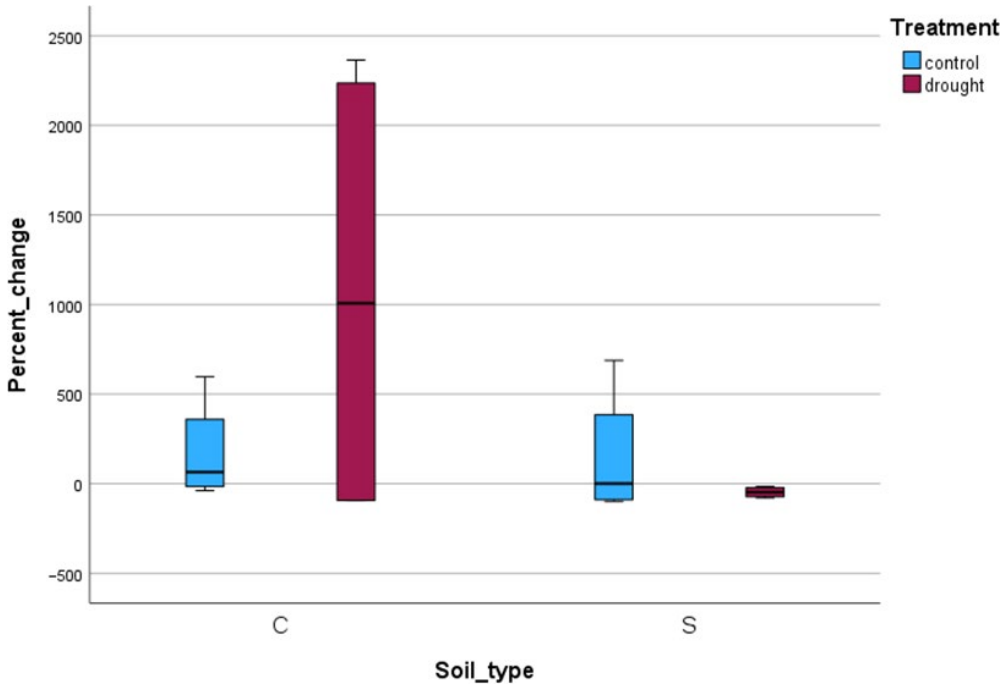

Figure 9: Boxplot showing the proportional change compared to starting material as percentage in fungal survival across soil type and treatment for eight fields from four farms. Each box represents a specific treatment paired with the soil type (Clay - Control, Clay - Drought, Sand - Control and Sand - Drought).

The boxplot presents the mean percentage change in DNA quantity for each soil type and treatment, relative to the starting material. The median values, which indicated increase or decrease of fungal DNA, for each group were as follows: clay-control: 65.4%, clay-drought: 1007%, sand-control: 0.5%, sand-drought: -47%.

The amount of fungal DNA remained largely unchanged in sand under control conditions, while drought appeared to reduce it. In clay soil, fungal DNA increased in both treatments, with the drought condition showing a substantial increase.

The qPCR for the 51 DNA extractions were conducted in 3 plates. Each plate included the serial dilution to build the standard curve, the samples used as control (i.e., the starting quantity of fungus when the kernels where freshly inoculated) and the experimental samples (i.e., the DNA extracted from the kernels after four months in the pots).

Plate 1 qPCR Results

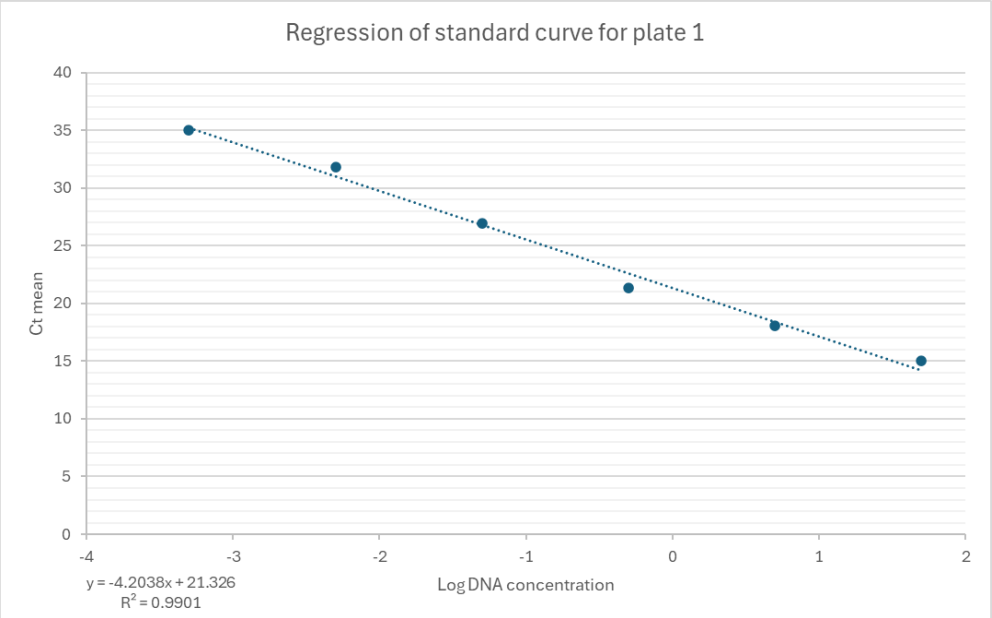

Figure 10: Plot graph depicting the serial dilution for plate 1.

The dots represent the standard curve. This plate had an R^2-value of 0.99 which indicates a strong correlation between the DNA concentration in the samples and the mean Ct value.

About eight of the samples on this plate had a standard deviation from 1.6 to as high as 4.0. The samples that showed a significantly high standard deviation were retested at a later date. However, due to inconsistencies for the fourth plate that was ran, it has been decided that the results will be excluded from this study. This will be further explained in the discussion.

Table 5: Table showing Ct mean and standard deviation of the samples of plate 1.

Sample	Ct mean	SD
A. solani SC	16.4	1.868348362
A. solani SC	18.1	0.39599269
A. solani SC	21.1	0.396989011
A. solani SC	27.5	1.381581433
A. solani SC	32.8	1.593384511
A. solani SC	35.6	0.271412271
Starting material	19.9	0.711922026
15	26.3	8.547855925
17	20.3	2.902959564
24	21.7	0.21361571
27	19.6	0.415469104
48	33.1	2.31887671
57	20.4	1.186719311
61	20.8	1.227414329
67	28	0.789274807
74	24.6	2.252267895
77	27.5	4.085588679
82	28.2	3.318215822
94	31.3	0.203958931
99	20.8	0.900912392

Plate 2 qPCR Results

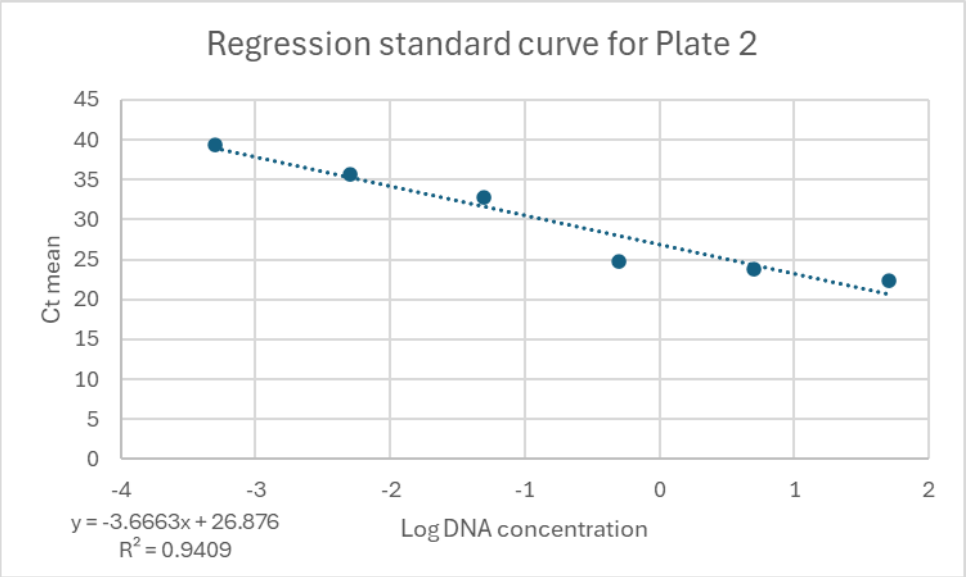

Figure 11: Plot graph depicting the serial dilution for plate 2.

Plate 2 analyzed the samples from plate 2. The graph had an R^2-value of 0.94 which indicates a high correlation between the DNA concentration in the samples and the mean Ct value.

However, the amount of experimental samples with a high standard deviation (> 1.7) on this plate was 10 out of 19 samples or approximately 53%, which is the highest out of all the plates. Despite the high variability among the experimental samples, the strong correlation as observed in the linear relationship between DNA concentration and Ct results suggests that the primers and qPCR performed as expected.

Table 6: Table showing Ct mean and standard deviation of the samples of plate 2.

Sample	Ct mean	SD
A. solani SC	24.8	0.9
A. solani SC	32.8	3.5
A. solani SC	35.7	2.1
A. solani SC	39.4	0.2
Starting material	24.8	0.2
Starting material	29.3	3.0
84	27.4	3.8

89	24.1	1.5
91	33.9	1.4
93	33.8	3.8
9	28.6	5.4
14	28.2	0.8
16	21.0	1.9
21	29.7	1.3
28	28.3	3.8
29	21.7	1.5
31	29.6	0.6
64	27.3	2.0
66	26.7	3.4
71	24.2	0.6
78	23.6	1.1
79	26.2	1.5
81	31.3	5.4
87	32.7	2.1
95	36.3	2.3

Plate 3 qPCR Results

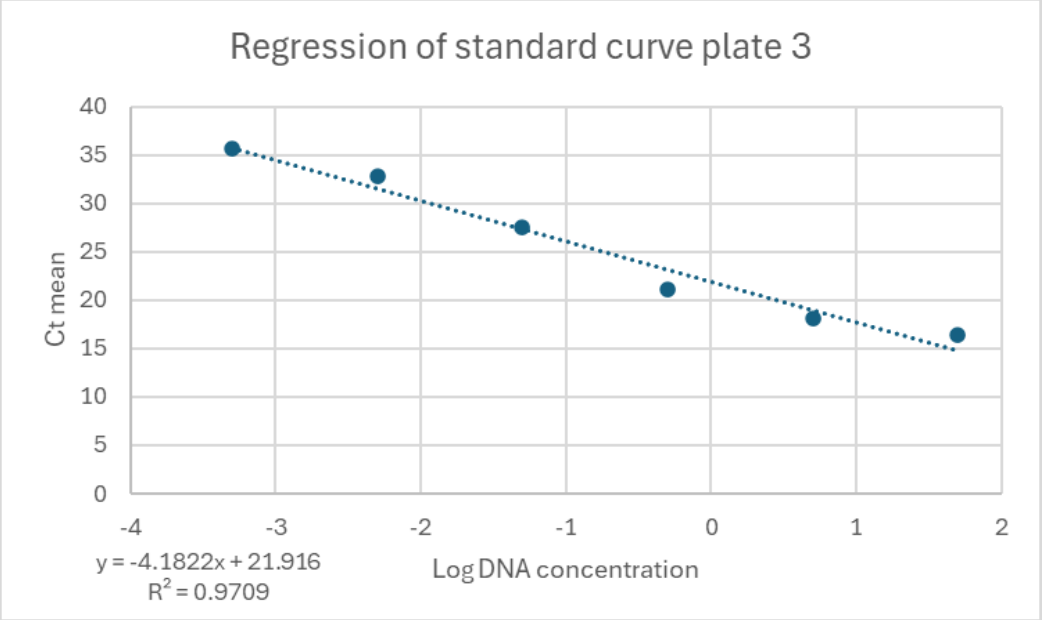

Figure 12: Plot graph depicting the serial dilution.

Plate 3 analyzed the samples taken from plate 3. The qPCR showed a strong linear correlation between the Ct mean and the logarithmic DNA concentration with an R^2-value of 1.

However, the variability between samples was notable. Out of the 13 samples analyzed, six of them showed a high standard deviation (> 1.7) with the highest being 8.55. This suggests inconsistency in the replicates analyzed.

Table 7: Table showing Ct mean and standard deviation of the samples of plate 3.

Sample	Ct mean	SD
A. solani SC	16.4	1.868348362
A. solani SC	18.1	0.39599269
A. solani SC	21.1	0.396989011
A. solani SC	27.5	1.381581433
A. solani SC	32.8	1.593384511
A. solani SC	35.6	0.271412271
Starting material	19.9	0.711922026

15	26.3	8.547855925
17	20.3	2.902959564
24	21.7	0.21361571
27	19.6	0.415469104
48	33.1	2.31887671
57	20.4	1.186719311
61	20.8	1.227414329
67	28	0.789274807
74	24.6	2.252267895
77	27.5	4.085588679
82	28.2	3.318215822
94	31.3	0.203958931
99	20.8	0.900912392

qPCR efficiency

The qPCR efficacy was calculated using the slope from the standard curve of each plate, using the formula $Efficiency\ (\%) = (10^{(-1/Slope)-1} \cdot 100)$.

Table 8: *The efficiency percentage calculated from the slope values of each standard curve.*

Plate code	Slope	Efficiency
Plate 1	-4.20	73%
Plate 2	-3.67	87.3%
Plate 3	-4.18	73.5%

These results are quite low considering the desired value for the qPCR efficiency is between 90% - 110%. The lower efficiency may be attributed to pipetting inaccuracies or inconsistent sample handling, contaminants in the DNA extracts and primer binding, all of which can negatively affect the amplification process.

Despite the lower efficiency, the results may still be useful for qualitative analysis regarding the survival and increase or decrease of *A. solani* compared to the controls. Overall trends and relative difference between groups tend to be preserved even when quantification is less reliable. Caution should be taken for analysis of individual samples or for quantification purpose.

Discussion

Germination experiment

The results for H.23.2.3.1, H.23.2.2.1, H.23.1.1.2 and H.23.1.3.4 show spore germination being lower for concentration C_5 compared to C_6. The differences range from 1% to 5%. This can be explained due to handling related variations such as pipetting errors during isolate transfer or spore concentration rather than indicating a reduced efficacy of the treatment at the highest concentration.

Another reason for this can be due to the mean germination count being calculated using only one of the duplicates rather than both. For isolates H.23.2.3.1 and H.23.1.3.4, both of which had a lower spore germination for C_5, only one plate could be used due to low spore count. H.23.2.3.1 had the highest difference between C_5 and C_6 with a 5% difference. This factor likely explains the unexpected results for both isolates. Samples H.23.2.2.1 and H.23.1.1.2 also had to rely on one duplicate to calculate mean germination percentage. For C_0 and C_4, respectively. The exclusion of duplicates may have reduced the reliability of the germination percentage estimate for concentrations in question.

It is also worth noting that none of the isolates showed a complete stop in germination at the highest concentration, C_6. This suggests that while fluopyram was effective in reducing germination rates at high concentrations, it did not fully inhibit spore viability. This might indicate difference in sensitivity among isolates, which is also supported by existing literature (Lefèvre, 2023). The referenced study observes that sporulation in the isolates was influenced by factors outside of fungicide concentration, such as potential biological variation. Although these aspects were not the focus of this study, they may be valuable areas for future research to explore.

These findings align with existing literature where fluopyram showed significant reduction of mycelial growth for *Alternaria solani* (Ekdahl, 2022). While the referenced study did not focus on spore germination specifically, given that spore germination is a prerequisite for

mycelial growth, the results support a similar conclusion: Fluopyram demonstrates a strong inhibitory effect on *A. solani* suggesting that it is still efficient to control A. solani in the fields..

Isolation experiment

As shown on Table 4, the qPCR confirmed the presence of Alternaria in all samples, indicating successful isolation and detection. This result confirms the effectivity of the isolation protocol. In the untreated leaves, presence of both *A. solani* and *Alternaria spp.* were confirmed, meaning both species coexist in the field.

As mentioned in the results, *Alternaria spp.* dominated the samples treated with fluopyram, with some of the samples consisting of only *Alternaria spp.*. This result may suggest other Alternaria species, such as, *A. alternata* is less sensitive to fluopyram than *A. solani* or that interspecies dynamics change with application of the fungicide.

Even though the Lyckeby strategy was not the main focus of the study, the samples were analyzed for future research. These results varied the most, with *A. solani* occupying approximately 10% at its lowest and up to 70% of the samples. *Alternaria spp.* occupied 30% at its lowest and 90% at its highest of the samples.

Throughout the process, a few technical limitations were encountered. One of the samples, #19, did not have the proportion of *A. solani, Alternaria spp.* and "others" noted. However, this does not affect the analysis as no average was calculated for this data.

On step 3 of the isolation, a few samples had to be discarded due to contamination, likely bacterial growth, or lack of *A. solani* or *Alternaria spp.* presence. Due to a labelling error, two samples were marked as #6. In order to differentiate them while retaining their data, they were marked as #6.1 and #6.2. However, the original leaf and treatment group can not be confidently traced back as a result of this relabelling. Therefore, this could affect the interpretability of the result for those specific samples.

Overall, the results of the isolation experiment indicate the presence of *Alternaria* species in all samples, albeit at varying proportions. While levels of *A. solani* were expected in the treated samples, it is noteworthy that many treated samples contained *Alternaria spp.*. This may indicate a lower sensitivity of *A. solani* to fluopyram while other species may be less affected.

Soil experiment

As seen in Figure 5, the highest increase in fungal survival was observed in the drought-treated clay soil, which showed the greatest increase in DNA quantity compared to the starting material. It was also the group with the highest variation as notable in the plot. Meanwhile, sandy soil under "control" conditions showed a negative median value, as well as the least variation out of all the groups.. This suggests that the clay soil under dry conditions

may be most favourable for Alternaria solani survival. Sandy soils, being more porous, might provide challenges for pathogen inoculation due to environmental stress. Clay, being able to retain moisture, combined with reduced rain, may have created a more stable environment that favoured pathogen survival.

The clay soil and drought combination also showed the greatest variation out of all groups. This could be attributed to biological diversity between samples, inconsistencies in handling and small environmental differences. For example, under dry conditions, clay-rich soils may dry unevenly. This could lead to higher variability in fungal survival, even within the same soil. The dry conditions might reduce competition between organisms, and if *A. solani* is favoured this could promote the growth of the pathogen. These findings align with existing literature where *A. solani* survived better in dry soil (Jindo et al. 2021) as well as results from a study by Lefèvre (2023) where *A. solani* infected kernels were both weighed and analyzed using qPCR to study the survival of the pathogen. Clay soil coupled with dry conditions lead to better outcomes for *A. solani* survival.

In comparison, Stridh et al. (2023) reported contrasting results, where higher sand content was associated with higher risk for severe infection. These results were linked to the increased likelihood of potassium deficiencies in sandy soils, which in turn increased susceptibility to the pathogen (Stridh et al. 2023). This highlights the role soil conditions, such as texture and nutrient availability, play in infection risk, showing the complexity of assessing A. solani management.

Due to the high standard deviation in some of the samples, we decided to rerun them on a fourth PCR plate. However, the fourth plate yielded limited information, with many samples producing no values (NaN). This was likely caused by low DNA concentrations as it was observed that several samples were low in volume. Therefore, the results from previous plates were used for analysis in order to retain as much information as possible.

Conclusion

This study highlights challenges posed by *Alternaria solani* in cultivation of potato and the future obstacles we may face. While fluopyram was successful in reducing spore germination, complete inhibition was not achieved. The pathogen was still present along with an unexpected proportion of *Alternaria spp.* present on treated samples. Furthermore, the soil experiment demonstrated the pathogen's ability to survive in dry clay soils - a factor that will become increasingly relevant for the future as we face warmer and drier climates. These findings emphasize the need for ongoing research, monitoring fungicide efficacy and integrated disease management to ensure the sustainable use of fungicides.

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