Inoculation methods for pathogenesis of *Venturia inaequalis*

– for screening *Malus* spp for resistance.

Berglind Hreiðarsdóttir

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Swedish University of Agricultural Sciences, SLU
Horticultural Management Programme - Gardening and Horticultural Production (BSc)
Faculty of Landscape Architecture, Horticulture and Crop Production Sciences
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Supervisor: **Kimmo Rumpunen**
Examiner: Jonas Skytte av Sätra

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Abstract

The purpose of this study is to get an overview of how plant breeders can screen genotypes of *Malus spp* for resistance against apple scab or black spot disease. This is done by inoculating the pathogen *Venturia inaequalis* onto apple tree seedlings and their symptoms are monitored and graded. The focus is on different application methods that are trialled to get a more physical understanding of the entire process. Factors to consider are which methods of applications are successful, simple, and repeatable. A literary study is done on the current and past methods available, and the methods are compared to get an overview of the pathogen's requirements to successfully establish an infection on the host in an experimental setting. Along the literary study, four application methods are tested on young apple trees to get the practical understanding of what a screening entails and what the various methods demand. The result of the experiment will mainly consider the practicality and applicability of each method that is successful.

Keywords: Venturia inaequalis, apple scab, *Malus*, foliar inoculation, methods, resistance, screening, selection, plant breeding, skorv, resistens, växtförädling, selektion.

Abstrakt

Syftet med arbetet är att göra en litteratursammanställning kring metoder för inokulation av skorv på fröplantor av äpplen samt att utvärdera några av metoderna. *Venturia inaequalis*, en svamp som orsakar äppelskorv. Äppleskorv är ett globalt problem och orsakar stora förluster hos äppelodlare, speciellt i områden med svala fuktiga vår. Eftersom fungicider har sin miljöpåverkan och svampen kan bygga upp resistens blir det mer aktuellt att förädla fram sorter som har resistens mot svampen. För att kunna utveckla nya äpplesorter med hög motståndskraft mot skorv behövs enkla systematiska, effektiva och upprepbara screeningsmetoder som kan användas vid selektion av potentiella nya plantor. En litteratursökning kring olika metoder för skorvinokulering genomförs samtidigt som ett experiment med fyra utvalda metoder genomförs på äpplefröplantor i växthus. För inokulering används skorvinfekterade blad insamlade hösten 2023. Experimentet utvärderar effekter av skorvinokulering, resursbehov och tidseffektivitet.

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Abbreviations

Avr	Avirulence
ECPGR	European Cooperative Program for Plant Genetic Resources
GfG	Gene for gene
HPI	Hours Post Inoculation
MA	Malt Agar
RH	Relative Humidity
PDA	Potato Dextrose Agar

Wordlist

Adaxial	The upper side of the leaf
Axenic	A state of culture that is free from any contaminants.
Genotype	An entire set of genetic material of an organism.
Germplasm	Any part that can be used as a genetic resource.
Inoculum	Source material used for inoculation (an act of implanting one organism to another).
Ontogenic resistance	Ability of plants, or parts of it, to get more resistant or tolerant against disease as they mature.
Scab race	Population of scab individuals that share a combination of same virulence genes.
Virulence	Ability of the pathogen to cause damage to a host.

1. Introduction

1.1. Background and context

The fungus, *Venturia inaequalis* (Cke.) Winter, is responsible for apple scab disease, one of the most commercially significant pathogens in apple, *Malus domestica* Borkh., cultivation (Khajura et al., 2023). The infection occurs in leaves, branches, fruit, and flowers. Leading to lack of quality and quantity in production. The fruit develops a cracked, deformed appearance (Belete and Boyraz, 2017) and the infection can also lead to unripe fruit fall (Agrios, 2005). The disease is managed in cultivation by frequent fungicide spraying each year (Sokolova and Moročko-Bičevska, 2022b). In Sweden 2024, there are eight fungicides that can be used against scab and the maximum quantity of treatments ranges from two to six times per season (Zandelin et al., 2024). In central Europe the fungicide applications can reach 20 – 25 times depending on conditions each year (Patocchi et al., 2021). Not only does this promote the development of *V. inaequalis* strains with fungicide resistance but also does the recurring application have detrimental environmental factors and possible health problems for workers (Khajura et al., 2023). To minimise use of fungicide and develop a more sustainable controlling of apple scab, plant breeders are opting for developing cultivars with scab resistant genes (Sokolova and Moročko-Bičevska, 2022a; Belete and Boyraz, 2017).

To test the resistance of seedlings varieties it is important to have a reliable method for screening them. This involves foliar inoculating the plants with the fungus *V. inaequalis* and closely monitoring both infestation and spread. Diverse methods have been employed for this purpose. The approaches that are often inadequately described lead to complications with comparing results. For this reason, this study includes both a literature review and an experiment.

1.2. Aim

The literary review aims to provide a comparison and an overview of the methodologies used to inoculate *V. inaequalis* onto its host and which other measures need to be considered to ensure pathogenesis. To deepen the understanding of the practical aspects; an experiment will be conducted trialling four methods on four leaves of the test subjects. Performing the inoculation requires host and pathogen material, and a place to perform the experiment. Deciding on conditions, methods and the grading of symptoms are also important factors.

1.3. The Pathogen and limitations

In the process of collecting and producing the pathogen for the experiment it is adamant to know the pathogens reproductive morphology in its different stages. This to ensure vitality and thereafter proper infection of the pathogen material. When grading the infection, it is also important to familiarise with the early symptoms of the disease. The various strains of apple scab are usually referred to as races. These can differ morphologically and physiologically. Race 1 was the most common in Sweden twenty years ago (Sandskär and Liljeroth., 2005). Breeding programs for resistant cultivars has since then perhaps shifted the concentration of the more common races. The pathosystem between *Venturia inaequalis* and *Malus* is described as a Gene for Gene (GfG), a form of recognition (Khajuria et al., 2018). The resistance gene in the host causes a avirulence (A*vr*) of the host. Leading to the host activating a defence response. This relationship can be important when designing a screening method as the resistance characteristics are on a gene level depend on both the source of host and pathogen. This study will not focus on the races and/or a particular isolate of scab but rather include a sample of unidentified scab races from the local area. Since the aim is merely to get familiar with the practicality of preparing and applying the inoculum. The host material, the apple tree seedlings, are also of various origins. Hence why the target area for each method is a leaf of an individual and not the whole plant.

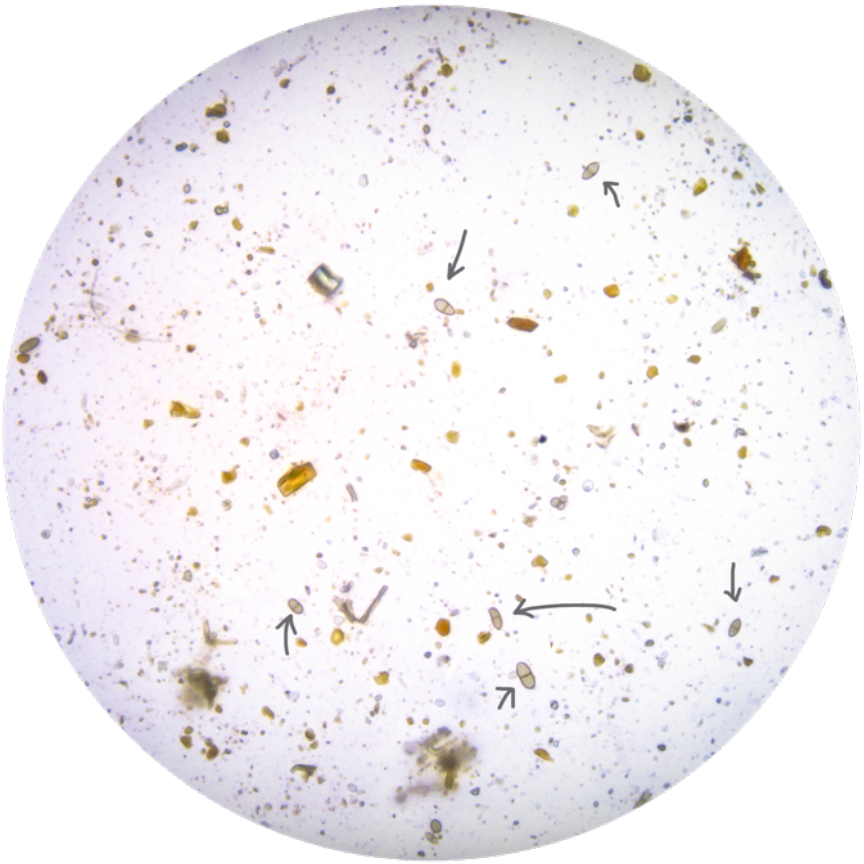

Figure 1. Ascospores from leaf suspension (Hreiðarsdóttir 2024).

1.3.1. Spore Morphology

V. inaequalis has a dual reproductive system and therefore two different forms of spores. The ascospores (5 - 7 µm wide and 11 - 15 µm long), a footprint shaped two celled spores (see figure 1), are released in springtime from the asci, containing 8 spores each (Gauthier, 2018). The asci mature within the pseudothecia, a tiny dark dome like fruiting body on fallen leaves. The conidia (6 - 12 µm wide and 12 - 22 µm long), the asexual form is single celled and develops from the established lesions. Both spores penetrate the cuticula and establish infection around the epidermal cells expanding their mycelium mostly in the subcuticular areas (see figure 2).

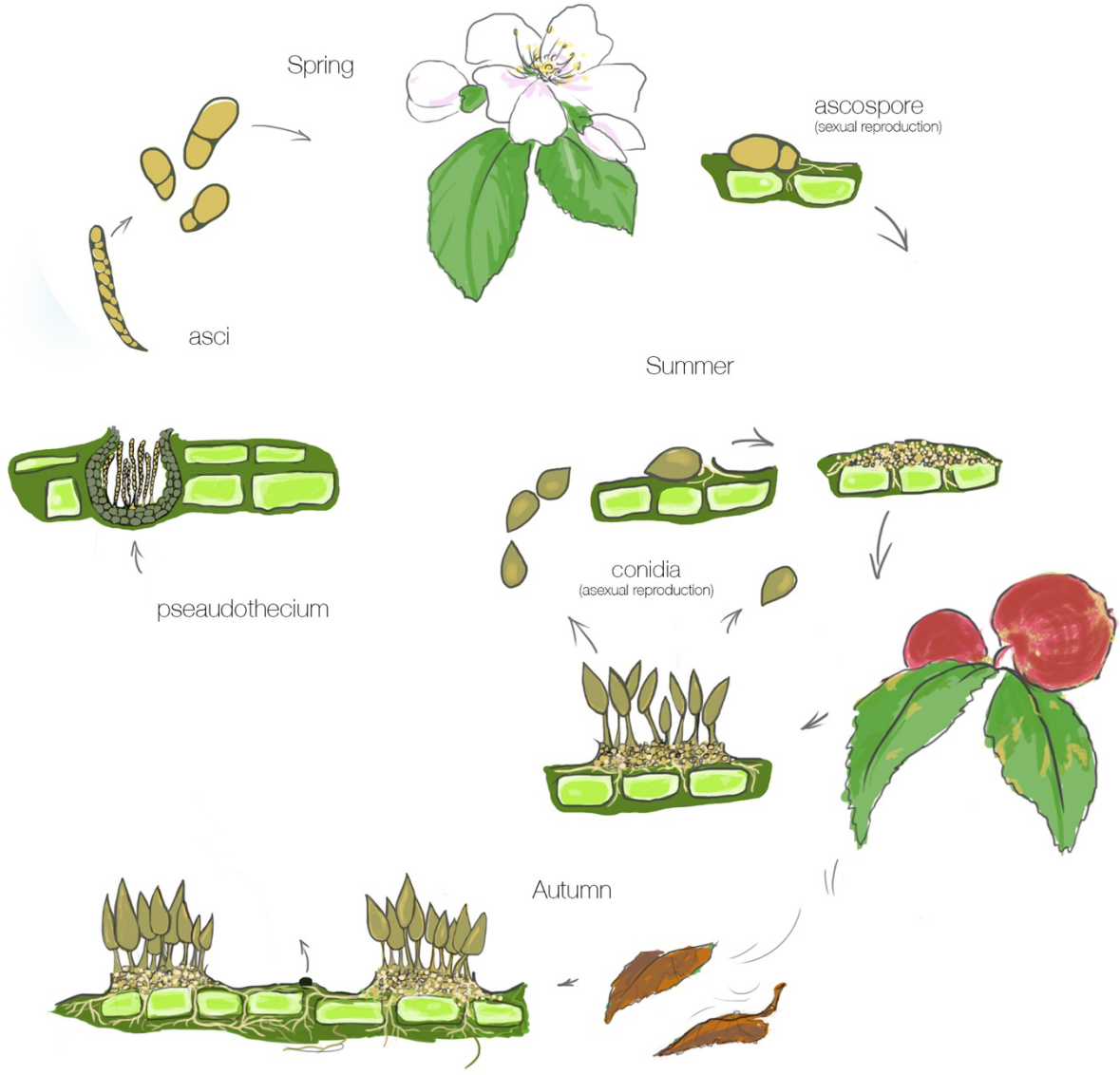

Figure 2. Lifecycle of Venturia inaequalis (Hreiðarsdóttir 2024).

1.3.2. Apple scab Lifecycle

V. inaequalis is an ascomycete fungus and hemibiotroph; meaning it has a dual lifestyle (see figure 2). It lives on the hosts live cells in spring and summer taking up nutrients for its survival. During fall/winter the pathogen turns to a necrotic lifestyle where it takes up nutrients from dead cells. Overwintering in fallen leaves the pathogen releases ascospores (sexual) in wet conditions around springtime (Gauthier, 2018). The ascospores land on the newly emerging leaves establishing primary infection. (Sokolova and Moročko-Bičevska, 2022b; Sandskär, 2003). From there the pathogen can reproduce asexually via conidia throughout the season. The overwintering conidia found in buds and pustules on young shoots can also be a source of primary infection (Rancāne et al., 2023).

Figure 3. Apple scab symptoms on Malus prunifolia (Hreiðarsdóttir 2024).

1.3.3. Symptoms

The macroscopic symptoms appear in around 10 days on the leaf, small irregular spots in light olive green (see figure 3) (Agrios, 2005). High humidity along with leaf wetness for several hours is needed for the pathogen to establish a stroma and later sporulate (Gauthier, 2018). As it spreads it turns a more greyish tone with a velvety texture and later as the pathogen matures it becomes darker, metallic and the texture might be blistered as seen in figure 4. The fruit symptoms develop similar velvety olive-green lesions at first before they take on the signature scabby appearance. A characteristic halo, lighter or pinkish shade, often surrounds the dark lesion. The scruffy shapes can result in distortions and cracks in the flesh (Gauthier, 2018). Symptoms of resistance can be tissue chlorosis or necrotic patches without sporulation (Sokolova and Moročko-Bičevska, 2022b). Bus et al. (2011) described three sets of qualitative resistance characteristics. A hypersensitive response; which looks like scattered pin points that are barely visible. A stellate shaped necrosis. The last one being chlorosis with limiting sporulation.

Figure 4. Apple scab symptom starts out olive green and later turns darker and metallic (Hreiðarsdóttir 2024).

2. Material and Method

2.1. Literature

A literary review is conducted to get an overview of the current and past methods used to inoculate apple scab onto *Malus* spp. older studies, such as the one performed by Chevalier et al. in 1991, generally have a more descriptive methodology which is why they are still relevant. Key considerations in selecting relevant research papers include the origin of the fungi, as in where it was collected and the conditions under which it was stored prior to application. Which condition the experiments took place. The stage of the plants during the experiments. The inoculation method of the fungi onto the plants. Finally, the grading of the symptoms or the evaluation step. To compare the different methods tables 2 – 8 were made with the various factors mentioned. While information in table 8 containing results is less comparable due to the various objectives of each study, it still can give an insight into the research fields on apple scab inoculum. Blank ('-') spaces within the tables signify lack of information.

2.2. Experiment

The host material for the pathogen, various *Malus* ssp seeds were sown 06.02.2024. They grew in a controlled environment until at least six leaves developed to their mature size on the plant. According to Belete and Boyraz (2017) the infection risk is mostly prominent at the start of the season on the susceptible young leaves making the experiment on seedlings a good option for screening. Controlling the spread or pressure of the pathogen would also be difficult to manage in a field setting. Dry fallen leaves from the previous season were used as a source material for the inoculum. The leaves were collected around infected *Malus* spp in Alnarp. The leaves were macerated with water and ascospores were counted with a Bürker counting chamber. The spore suspension was then mixed as seen in Table 1, making the final concentration 3.8 x 10^4 ascospores per mL. Ten plants were selected for the experiment and each of the leaves number 3 – 6 (counted from first true leaves) got a different treatment as seen in figure 5. Comparing the different treatments on the same genotype was important due to the variation with seed material. Each plant was put in an enclosed bucket to keep up the humidity during the incubation period.

The incubation time was 65 hours, and the buckets were placed in a windowless room temperature setting for a more stable environment. Post incubation the plants were placed back in the greenhouse. After a hot day on the greenhouse bench, the plants were moved back into the buckets for them to remain in a more humid cooler environment as seen in figure 6. However, this time the lids were replaced with see-through plastic bags to enable the light to reach them as well. The buckets remained in the shady part of the greenhouse. The plants were monitored for a month and were checked for symptoms around three times a week.

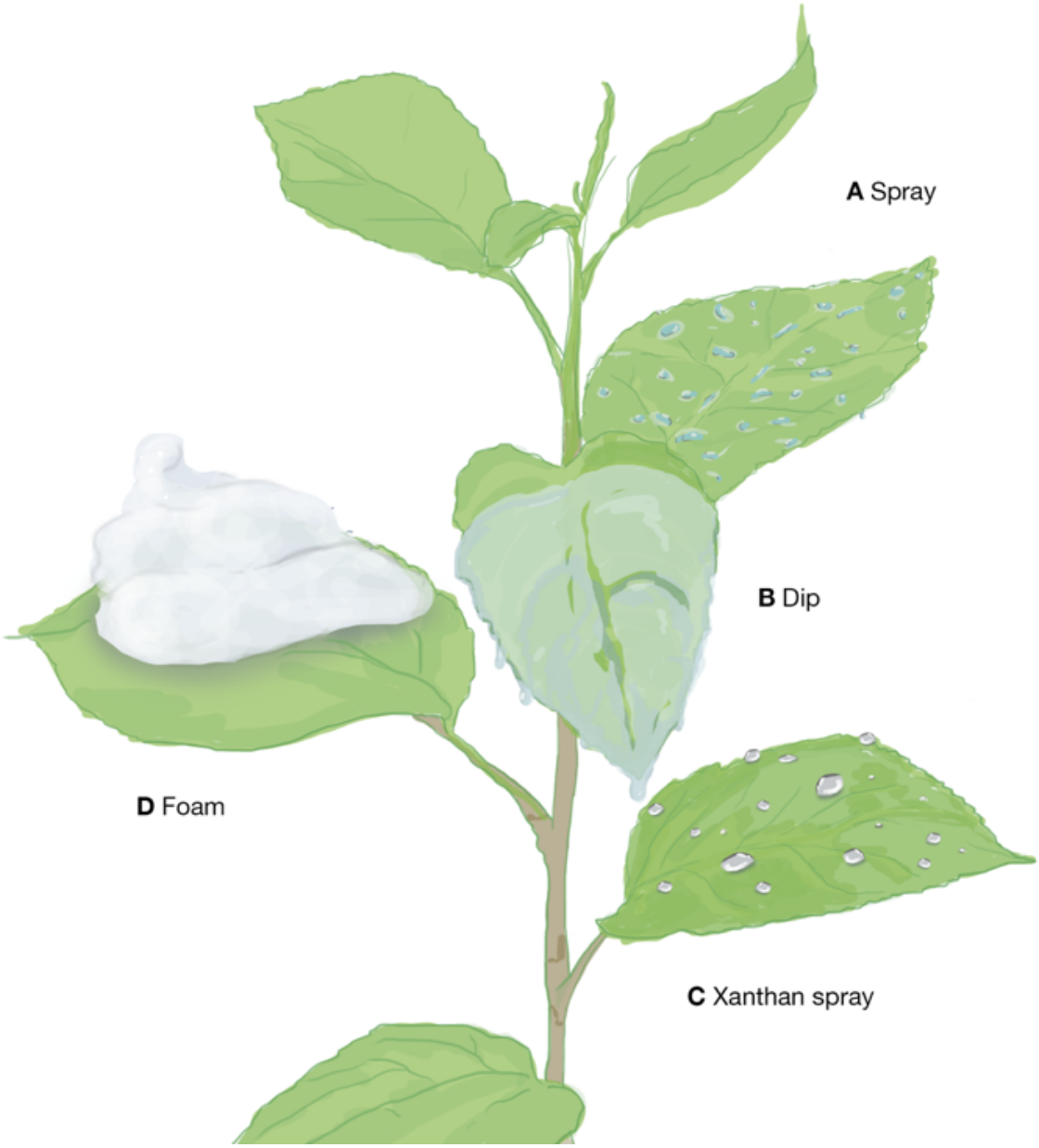

Figure 5. Chosen methods for the experiment. (Hreiðarsdóttir 2024).

Table 1. Experimental treatments for test of inoculation of ascospores of Venturia inaequalis on apple seedlings in controlled conditions.

Spore suspension $1x10^5$ ascospores per mL Water		3g XANTURAL 75 Powder (CP Kelco, US) to 60ml water	
Spray **A**	Dip **B**	Xanthan spray **C**	Xanthan foam **D**
22.5 mL spore suspension + 37.5 mL water	22.5 mL spore suspension + 37.5 mL water	Mix 22.5 mL spore suspension + 30 mL water + 7.5 mL xanthan solution	Whisk 22.5 mL spore suspension + 30 mL water + 7.5 mL xanthan solution

A. The spray method. The diluted spore solution is sprayed onto the leaf with a handheld sprayer.

B. The dip technique. A leaf is dipped into the diluted ascospore solution.

C. The xanthan spray. Xanthan gum powder is mixed with water and then mixed with the diluted spore solution. The mix is then added to a handheld sprayer and applied to the leaf.

D. The foam technique. Xanthan gum is whisked together with the spore concentrate which is applied with a spoon onto the leaf.

3. Literary review findings

To screen *Malus* germplasm for their susceptibility or resistance against apple scab it is imperative to have a standardised method of testing ensuring that the screening results will be comparable between years. Steps for artificial inoculation is as follows: 1) Preparation of inoculum. 2) Creating suitable environment for host and pathogen. 3) Treatment of plants. 4) Grading the symptoms.

Table 2. Inoculum material, the source material for their lab culture or where they collected it (if mentioned). Or which parts of the Malus spp the spores were collected from. Conidial suspension refers to the amount of conidia per mL used for the inoculum. Spore storage refers to if the infected leaves were stored, sometimes the studies mention cultivating the spores before storage.

Ref	Spore source	Conidial Suspension	Spore storage
1.1	Strains HS1 and SW01.	5 x 10^4 conidia/mL	Stored on leaves at - 20°C.
2.1	Race = 0.	5 x 10^5 conidia/mL	-
3.1	9 strains Latvia, Poland.	-	-
4.1	-	5 x 10^5 conidia/mL	-
5.1	Lesions on leaf, R2.	1.5 - 2.5 × 10^5 conidia/mL	Cultivated and stored on dry cellophane membrane (see table 3) at -20 °C.
6.1	Diseased organs of various *Malus spp.*	1 × 10^6 conidia/mL	Frozen -18 °C.
7.1	Neighbouring plants.	-	-
8.1	Kashmir valley.	5 x 10^5 conidia/mL	-
9.1	Washed from leaves.	5 x 10^6 conidia/mL	-
10.1	Young lesions, mixed orchards.	3 × 10^5 conidia/mL	Dried and frozen -20 °C.
11.1	Naturally.	-	-
11.2	Scabbed leaves, Alnarp.	2-3 x 10^5 conidia/mL	Frozen and fresh used.
11.3	Naturally.	-	-
12.1	Leaves, Belgium.	1.5 x 10^5 conidia/mL	Dried for a week, frozen -18 °C.
13.1	-	2 x 10^5 conidia/mL	-
14.1	Fruit, 'Royal Gala'.	1.5 x 10^4 conidia/mL	-
14.2	Naturally.	-	-
15.1	-	10^6 conidia/mL	-
15.2	-	10^6 conidia/mL	-
15.3	-	10^6 conidia/mL	-

3.1. Preparation of inoculum

For an artificial inoculation the spore material is usually collected from infected leaves in orchards. There are also examples of using scab lesion from fruit such as

Li and Xu (2002) did for their experiment on older leaf susceptibility to scab. Some studies such as Chevalier et al. (2004) just mention the final concentration.

The suspension concentration generally used is around 5×10^5 conidia/mL. The suspensions can be non-axenic, as in not completely isolated from other matter, washed of the leaves and mixed with water and sprayed directly onto the host (Yepes and Aldwinckle, 1993). Doolotkeldieva and Bobusheva (2017) used a sample of five parts of the plant displaying symptoms which was macerated and slowly poured and/or filter in a tube to leave the pulp. The sample was then centrifuged before placing on a petri medium for establishment. Sandskär and Liljeroth (2005) filtered their leaf solution after maceration through a cheesecloth, isolate monoconidial strains and then cultivated on MA. Some studies mention the use of antibiotics when cultivating their pathogen (Steiner, 2023; Chevalier et al., 2008). Barbara et al. (2008) explains how to use a cellophane technique to produce conidia *in vitro*. Storing entails drying and freezing across studies, this includes both leaves and/or cultured samples for example on cellophane membranes.

Table 3. Pathogen cultivation. Some studies grew their conidia for their inoculum on petri plates with a medium, usually Malt agar (MA) or Potato dextrose agar (PDA). Below is a short description of their methodologies.

Ref	Spore cultivation
1.1	Conidia washed of diseased leaf, grown on PDA, MA and incubated for 2 weeks with antibiotics, 18°C.
2.1	-
3.1	Refer to own previous work on inoculum, previous work not found.
4.1	Pure culture.
5.1	Conidia picked w needle after 24 hours, put on MA and kanamycin for 6 weeks. Then further cultivation on MA cellophane membranes.
6.1	A sample (5 parts displaying disease). Placed in 150 ml container with PO_4^{3-}buffer placed on rotary shaker and incubated 1-1.5 hours at 200 rev/min. Macerate, slowly pour into tube leaving pulp and or filtered. Centrifuged for 10 minutes at 8000 g and for 15 minutes at an acceleration of 7000 g, at a temp below 10°C. Supernatant discarded. Resuspended in 1 ml buffer, then grown on different mediums, PDA, Apple agar, Czapek's medium, Wort agar.
7.1	-
8.1	Single spore isolate cultivated, Inocula, refer to Barbara et al. (2008).
9.1	-
10.1	Cellophane soaked in RO for 20 hours. layer with filter in a Petri with water, cover with al foil and autoclave. Discs onto PDA malt extract (20 gL^{-1}) agar in Petri, no bubbles. Macerator, used to blend an isolate, 100 mg, from a PDA plate with 5 mL water for a few sec. Pipette on cellophane. Incubate at 18 °C (10/14 hours light/dark) 10 – 14 days.
11.1	-
11.2	Dilution from leaf, passed through cheesecloth, monoconidial strains isolated, cultivated on MA 18 °C in dark for 1 mth, transferred and subcultured for 3 w. Fungi incubated in flasks for 17 d in 1% malt extract, cheesecloth for oxygenation and 16h photoperiod. then frozen or used directly.
12.1	PDA 20°C - germination check.
13.1	Monoconidial strains of *V. inaequalis* and *V. pirina.*
14.1	Scab lesions washed with distilled water.
15.1	Axenic solution and non-axenic.
15.2	Centrifugation, 2000 x g for 5 minutes.
15.3	Non axenic.

3.2. Host

The leaves are the most usual spot to inoculate. Likely due to the ease of monitoring the symptoms. The leaves are most susceptible at a young age, this age-related resistance is called ontogenic resistance (Li and Xu, 2002). Li and Xu (2002) inoculated detached leaves (see table 4. Ref 14.1) where they compared old to young leaves and noted that the older leaves do get infected but show little to no symptoms and mycelia grows slower. Despite this information very few studies mention which stage the leaves are at point of inoculation, although Sokolova and Moročko-Bičevska (2022b), also working with detached leaves, mention it as a probable factor to their result (see table 4. Ref. 3.1 - 3.2). As seen in table 4 the host age varies from seedlings on their 3 - 4 leaf stage to 12 year old trees.

Table 4. The host material used in the different studies. Depending on what the studies mention, this could refer to parts of the tree or specific cultivars. Host age at time of experiment. Container or medium of the host material if mentioned in the study.

Ref	Host source	Host age	Container and/or medium
1.1	Seeds from *Malus x domestica,* cv. 'Cripps Pink'.	Seedlings with minimum 6 leaves.	Potted with standard substrate.
2.1	*Malus* 'Golden Delicious' and 'Royal Gala'.	-	-
3.1	*Malus* genotype, detached leaves.	Mature leaf.	Petri plates with moist filterpaper.
3.2	*Malus* genotype, detached leaves and unripe fruit.	Mature leaf, unripe fruit.	Petri plates with moist filterpaper.
3.3	*Malus* genotype, detached leaves.	Mature leaf.	Oasis in aluminium tray.
4.1	*Malus* 'Golden Delicious' grafted on 'M.9'.	1 year.	Potted.
5.1	Various *M. domestic*a cv. grafted on 'M.9'.	-	-
6.1	*Malus* 'Aychurok'.	Seedlings.	-
7.1	*Malus* 'Cox's Orange Pippin and 'Gala' on 'M.9' rootstock.	10 - 12 Year old.	Potted.
8.1	*Malus* trees - various cultivars.	1 Year.	Potted.
9.1	*Malus* 'Golden Delicious' grafted on 'M.9'.	3 Years old.	Peat substrate.
10.1	*Malus* 'Bramley', 'Cox' and 'Worcester'. [*sic!*]	1 - 2 Years old.	Potted.
11.1	13 Cultivars or *Malus* spp. grafted on B.9.	Planted in field, a year before trial starts.	
11.2	13 Cultivars or *Malus* spp. grafted on B.9.	-	-
11.3	13 Cultivars or *Malus* spp. grafted on B.9.	-	Potted.
12.1	Partially resistant, *Malus* 'Brown's Apple' and 'Mosancelj'.	Seedling 3 - 4 leaves.	Potted.
13.1	*Malus* 'Gala' and 'Discovery' grafted on MM.106. *Pyrus* 'Pierre Corneille' and 'Navarra' grafted on 'Kirchensaller'.	-	-
14.1	*Malus* MM.106 rootstock plant (main shoot).	Plant 1 year old, Leaves 4 - 100 day old.	Host plants in pots.
14.2	*Malus* 'Mondial Gala'.	3 Years old.	Hosts in sand bed.
15.1	*M. domestica* shoots.	-	Baby food jar.
15.2	*M. domestica* detached leaves.	-	Petri plates 1% agar.
15.3	*M. domestica* trees.	8 - 10 Year old.	Potted.

3.3. Environment for infection and development

As previously mentioned, the pathogen requires high humidity to enable infection. Which is why artificial inoculation usually takes place in a controlled environment such as growth chambers and greenhouses. Relative humidity (RH) is kept around 100% from 24 Hours Post Inoculation (HPI) to 72 HPI. Ensuring that the leaf maintains surface wetness for the pathogen to penetrate the cuticula (Chevalier et al., 2008; Sandskär and Liljeroth, 2005; Lefrancq et al., 2004). The RH is maintained sometimes by a growth chamber, a humidifier or covering the plants with a plastic bag. Post the incubation period the RH is around 80% (Sokolova et al., 2022b; Sandskär and Liljeroth 2005). Temperature is mostly kept at 18-20°C (Rather et al., 2023; Sokolova et al., 2022b; Mansoor et al., 2020; Sandskär and Liljeroth, 2005; Li and Xu, 2002).

Table 5. Environmental factors, placement refers to where the experiment took place. Temperature, and duration if mentioned. Relative Humidity (RH) during incubation of the pathogen and duration if mentioned. RH after incubation time.

Ref	Placement	Temp [°C] (duration)	RH during incubation	RH post
1.1	Greenhouse.	18 - 20 (16 Hours day)	100% For 48 hours.	50-65%
2.1	Glasshouse.	20	48 Hours, polythene dark cover 85%.	85%
3.1- 3.3	Climate chamber.	20 - 22 Day / 18 night	-	80%
4.1	Growth chamber.	18	Continuously irrigated.	-
5.1	Ventilated greenhouse.	18 Day / 15 night	48 Hours, cover with plastic bag.	70%
6.1	Greenhouse.	15	Enclosed in plastic for 2 days.	85%
7.1	Within rows of 15-year-old orchard for 3-5 weeks for establishing infection then moved to tunnel.		-	-
8.1	Glasshouse.	20	Placed in polythene structure 48h.	85%
9.1	Greenhouse w. UV- permeable plastic.		3 Days over-crown irrigated.	-
10.1	Glasshouse compartment.	20	High by means of cotton wool at base of stem and plastic sleeve around the plant.	-
11.1	Field.	-	-	-
11.2	Growth chamber.	18 - 20	Nearly 100%, cover, plastic.	80%
11.3	Potted, portable screening orchard.	-	-	-
12.1	Glasshouse.	19 - 21	90%.	70%
13.1	Glasshouse.	19 - 20	90% 72 Hours.	-
14.1	Glasshouse.	20	24 Hours cover, plastic bag.	70%
14.2	Outside by orchard.	20	-	70%
15.1	-	19	-	-
15.2	-	-	-	-
15.3	Glasshouse.	20	-	-

3.4. Treatment of plants

The usual way of applying *V. inequalis* is by means of spraying as it is simple and fast. It can be difficult to ensure an even coverage. Barbara et al. (2008) opted for enhancing the spraying technique covering each plant with a plastic sleeve and sprayed till runoff, the sleeves maintained high humidity and kept the plants isolated from each other. Lefrancq et al. (2004) had a custom spraying bench to inoculate their plants. Rather et al. (2023) used an atomizer, which is essentially a very fine sprayer. Sokolova et al. (2022b) mentioned brushing the solution with a soft paintbrush onto the leaves in another study on *V. pirina* or pear scab. However, the second most used method would be droplets via pipette also used by Sokolova et al. (2022b). These methods are effective when targeting specific areas or conducting smaller experiments. An experiment on five different methods tested with the ascomycete *Alternaria brassicae,* got good results by using a droplet method combined with an agarose dome on a specific target (Giri et al., 2013). While inventive, it demands more manual labour than is typically intended for a practical screening method.

Table 6. Application methods of the inoculum onto the host material. Other refers to if the studies mention other tools or further explanation of environment during the incubation period.

Ref	Application method	Other
1.1	Spray on adaxial side of leaf.	-
2.1	Atomizer.	Humidifier.
3.1	Droplets w. pipette.	-
3.2	Droplets w. pipette.	-
3.3	Paintbrush.	Wrapped in polypropylene bags.
4.1	Spray.	-
5.1	Youngest leaves sprayed then wrapped in wet paper.	Screen shaded windows post inoculation.
6.1	Sprayed to point of runoff.	Diffused light 16 hours per day.
7.1	Naturally.	By means of proximity to other infected trees.
8.1	Atomizer, till runoff.	Humidifier.
9.1	Spraying whole trees.	-
10.1	Fine handheld sprayer.	-
11.1	Natural - Untreated with fungicide or insecticides.	
11.2	Sprayed to point of runoff.	-
11.3	Natural - Untreated with fungicide or insecticides.	
12.1	Spraying bench 48h.	Transplanted outside post inoculation.
13.1	-	72 Hours post inoculation checked for appressoria; five foliar discs collected.
14.1	Sprayed.	-
14.2	Natural.	By means of proximity to other infected trees.
15.1 - 15.3	Sprayed.	-

3.5. Grading the symptoms

How to grade the symptoms varies in the existing literature depending on the purpose of the experiments. For this study the focus is on macroscopic qualitative grading techniques. Lefrancq et al. (2004) used a scale adaption based on Croxall et al. (1952) where 0 = no symptom; 1 = 1 to < 5%; 2 = 5 to < 10%; 3 = 10 to < 25%; 4 = 25 to < 50%; 5 = 50 to < 75% and 6 = 75 to 100%. Other mentioned the assessment key of Lateur and Popler (1994) or their own adaptions of it (Peil et al., 2017). A scale from 1 – 9, where 1 shows no visible macroscopic symptom and 9 exhibit maximum infection; fruits and leaves are black with scab and eventual leaf fall and fruit drop is imminent. The European Cooperative Program for Plant Genetic Resources (Lateur et al., 2022) also based their global assessment scale on this variation, but it is divided into the incidence and severity visually observed on leaf and or fruit. Time of evaluation also varies but a week after inoculation is usually where the first observation is made. Other studies used the Chevalier et al. (1991) grading system or a variation of it, where between 4 - 12 days post inoculation the symptoms are evaluated from class 1 - 4. Class 1. Hypersensitivity, show small barely visible pits, that do not enlarge with time (appear 4 - 6 days post inoculation). Class 2. Resistance is characterised by chlorotic irregular lesions with hints of necrotic edges (appear 7 - 10 days post inoculation). Class 3a. Weak resistance and Class 3b. Weak susceptibility, (also within 7 - 10 days mark). 3a includes mostly necrotic and some chlorotic lesions. Whereas 3b includes some clear sporulating lesions and the occasional necrotic lesion. Class 4. Susceptibility shows a homogenous sporulating lesion across most of the leaf surface.

Table 7. Grading of scab symptoms, when, if the first observation is made. Observation refers to how often the observation is made. It can also refer to tools used or if the observation was made macro or microscopically. Scoring refers to which system was used to measure the symptoms. Length of experiment if mentioned.

Ref	First obs.	Observation	Scoring	Length of experiment
1.1	7- 16 Days post infection.	Electron microscope.	-	-
2.1	-	Monitored for 21 days.	0 - 4 Chevalier et al., 1991. scale with. added class 5.	-
3.1	1-2 Weeks.	1 Per week.	0 - 4 Developed by Chevalier et al., 1991.	-
3.2	1-2 Weeks.	1 Per week.		-
3.3	1-2 Weeks.	1 Per week.		-
4.1	Pooled samples taken once pre, post 1d, 7d, 24d for gene expression analysis.			-
5.1	3 Weeks post inoculation.	Twice, 3 week and 4 week post inoculation.	0 - 4 Chevalier et al., 1991.	2013 – 2016.
6.1	Seedlings grown for 9 to 12 days post inoculation before leaves are removed and conidia washed off, then concentration of suspension determined by means of haemocytometer.			-
7.1	Incubate in polytunnel for two weeks before samples were taken.		Incidence and lesion density.	-
8.1	14 Days post inoculation.	-	0 - 4 Chevalier et al., 1991. 0, 1, 2, 3 = resistant and 3b and 4 = susceptible.	
9.1	3 Weeks post inoculation.	<30% Infected leaf = weak infection, >30% infected leaf = strong infection.		-
10.1	14 Days post inoculation.	Assessed for presence of sporulating lesions using hand lens.		3 + years.
11.1	3 Times each summer 2001 and 2002.		0 - 4 Chevalier et al., 1991.	1999-2002.
11.2	3 Weeks post inoculation.	Macro and microscope.	Leaves w. sporulation counted.	-
11.3	9th July 2002.	Twice summer 2002.	0 - 4 Chevalier et al., 1991.	2002.
12.1	-	0 = none; 1 = 1 to <5%; 2 = 5 to <10%; 3 = 10 to <25%; 4 = 25 to <50%; 5 = 50 to <75% and 6 = 75 to 100%.		Evaluated annually for 4 years.
13.1	-	-	Germination of appressoria in %.	Few weeks.
14.1	Disc (15 mm) examined under light microscope after some treatment. And visually *in situ*.		No. of colonies. Length (lm) of mycelia.	A season.
14.2				A season.
15.1	12 Hours post inoculation.	12 Hour intervals 72 Hour post, then day 5, 7, 10, 15 days post inoculation.	Surviving / necrotic.	-
15.2			Surviving / necrotic.	-
15.3			Susceptible vs Resistant.	-

Table 8. Reference to the studies used in the tables. The author and year sorted from newest to oldest. Names of the studies and a short description or detail from the result.

Ref	Author and year	Name of study	Result
1.1	Steiner and Oerke (2023).	A Melanin-Deficient Isolate of *Venturia inaequalis* Reveals Various Roles of Melanin in Pathogen Life Cycle and Fitness.	Melanin deficient apple scab less successful at infecting host.
2.1	Rather et al. (2023).	Combinatorial approach based on conventional and molecular methods for identification of scab resistance genes in apple germplasm of Jammu and Kashmir, India.	Identified scab resistance genes in several commercial cultivars.
3.1	Sokolova, Moročko-Bičevskaa (2022 b).	Screening of *Venturia inaequalis* virulence and resistance of *Malus* genotypes to apple scab using *in vitro* methodology.	About 80 % of the combinations tested were incompatible and expressed necrosis or/and chlorosis without pathogen sporulation or with limited sporulation.
3.2	Sokolova, Moročko-Bičevskaa (2022 b).		
3.3	Sokolova, Moročko-Bičevskaa (2022 b).		
4.1	Mansoor et al. (2020).	Quantification of polyphenolic compounds and relative gene expression studies of phenylpropanoid pathway in apple (*Malus domestica* Borkh) in response to *Venturia inaequalis* infection.	Supports previous studies that phenolic compounds are involved in defence response, host-pathogen interaction.
5.1	Peil et al. (2017).	Apple cultivar 'Regia' possessing both Rvi2 and Rvi4 resistance genes is the source of a new race of *Venturia inaequalis*.	Apple cultivar 'Regia', possessing both Rvi2 and Rvi4 resistance genes, is the source of a new race of *V. inaequalis.*
6.1	Doolotkeldieva and Bobusheva (2017).	Scab Disease Caused by *Venturia inaequalis* on Apple Trees in Kyrgyzstan and Biological Agents to Control This Disease.	Orchard experiments promising result regarding *Trichoderma viride* as potential biocontrol agent.
7.1	Passey et al. (2017).	The relative importance of conidia and ascospores as primary inoculum of *Venturia inaequalis* in a southeast England orchard.	Ascospore= main source of primary inoculum (c.80% in this orchard), meaning eliminating leaf debris should be retained. Conidia as primary inoculum should not be ignored.
8.1	Dar et al. (2015).	Distribution of Apple Scab Race Flora and Identification of Resistant Sources against *Venturia inaequalis* in Kashmir.	-
9.1	Holzapfel et al. (2011).	Differential gene expression in leaves of a scab susceptible and a resistant apple cultivar upon *Venturia inaequalis* inoculation.	Defence of the Vf-resistant cultivar 'Rewena', based on different mechanisms than defence response of the susceptible 'Golden Delicious', (*V. inaequalis* inoculation in the greenhouse led to relatively weak scab symptoms).
10.1	Barbara et al. (2008).	Virulence characteristics of apple scab (*Venturia inaequalis*) isolates from monoculture and mixed orchards.	Study showed that the virulence pattern to 'Bramley', 'Cox' and 'Worcester' [*sic*!] did not differ statistically between isolates from a mixed orchard and those from monoculture orchards.
11.1	Sandskär and Liljeroth (2005).	Incidence of races of the apple scab pathogen (*Venturia inaequalis*) in apple growing districts in Sweden.	Apple scab races 1-4 and 6-7 found in different parts of Sweden, race 5 was absent, race 7 first time observed.
11.2	Sandskär and Liljeroth (2005).		
11.3	Sandskär and Liljeroth (2005).		

12.1	Lefrancq et al. (2004).	Screening Method for Polygenic Scab Resistance within an Apple Breeding Programme: Relationship between Early Greenhouse Screening Test on Young Seedlings and Their Scab Susceptibility in Natural Field Conditions.	Proposing that up to 25% of the leaf surface covered by scab sporulation can be used as the greenhouse selection threshold.
13.1	Chevalier et al. (2004).	Host and Non-Host Interaction of *Venturia inaequalis* and *Venturia pirina* on *Pyrus communis* and *Malus* x *domestica*.	Only the cultivars susceptible to the compatible *Venturia* spp gave some symptoms.
14.1	Li and Xu (2002).	Infection and Development of Apple Scab (*Venturia inaequalis*) on Old Leaves.	Older leaves = small colonies, but conidia can infect at any age.
14.2	Li and Xu (2002).		
15.1	Yepes and Aldwinckle (1993).	Selection of resistance to *Venturia inaequalis* using detached leaves from *in vitro*-grown apple shoots.	Non axenic = shoots died in 3-4 d, axenic = susceptible vs resistant cultivars were distinguishable 6-8 weeks post inoculation.
15.2	Yepes and Aldwinckle (1993).		
15.3	Yepes and Aldwinckle (1993).		Visible symptoms developed faster on susceptible cultivars in greenhouse than *in vitro*.

4. Experiment results

Results of applying scab on the seedlings with four different methods showed no clear symptoms three weeks post inoculation. The initial suspension for the experiment was counted as 1 x 10^5 ascospores per mL, which was then further diluted to a final suspension of 3.8 x 10^4 ascospores per mL. Each of the four different methods came with their own advantages and challenges.

A. The spray method was easy to apply, it dried rapidly through and did not provide with an even coverage between the different plants.
B. The dip method demands picking up each sample that requires dipping, runoff bound to happen but that can be diverted back to solution holder, the coverage is even and reaches both sides of leaves.
C. The Xanthan spray adhered well to the leaf, but the solution was too thick to give an even coverage, the large size of the droplets did however keep the leaf moist much longer past the incubation time compared with previously mentioned methods.
D. The foam made a thicker application possible which was more visible it also provided the leaf with moisture for a longer time. It was applied with a spoon, which took longer time than other methods.

Despite the various application methods none resulted with scab symptoms that could be graded.

5. Discussion

Inoculating apple seedlings with *V. inaequalis* for screening of resistance is the current conventional method in research and breeding programs. Being less complex than molecular methods it requires a great deal of preparatory work and familiarity with the pathogen. The GfG pathosystem also means that the objective for which race of *V. inaequalis* the resistance should match, this might depend on where the screening is situated. The source material also has a say in which resistant gene the screening might be aimed at, since the resistant gene targets a specific gene in the pathogen. For this study we had various seed sown host material, and the pathogen was collected from leaves without noting their origin. With no specific genes targeted, the aim was to test the methodologies from preparing the inoculum to applying it onto a host and grade the symptoms. Due to the lack of symptoms the latter was not relevant to the experiment.

Collecting and maintaining the source material in a viable state before the application. Drying the diseased parts, if stored, seems to have a significant importance. Most studies mention drying and freezing (see table 2.). The first batch was picked in fall and stored in a refrigerator in a plastic bag with some moisture, the next two batches were picked from trees in spring and kept dry. When examining samples in a microscope there were issues finding viable spores in the first batch, the reason is not known. Familiarising with the morphological features of the pathogen took some practice. Counting the spores in a non-axenic solution with a Bürker counting chamber proved to have its challenges as well. The other studies mention conidia per mL. However, due to their small size and very uncharacteristic, single celled and basic shape; they were hard to identify amidst all other microbes and disintegrating plant tissue. Therefore, the counting of the two celled footprint shaped ascospores was more reliable (see figure 2). Also, perhaps more relevant when using fallen leaves as source material, where the ascospores stem from. The final count of ascospores in this case resulted in low quantities compared to other studies, which could also be a factor in why there were no symptoms.

All the studies mentioned conidia in their inoculum suspension and although never fully explaining the reasoning one can hypothesise on the matter. The inherent genetic variation of the ascospore, as it is sexually reproduced, could mean that it has a varied infection establishment success rate. Whereas using conidia, asexual reproduction. The certainty for successful infection of that specific gene material rises. Due to having infected a previous host, as it is a clone of an already established infection.

Figure 6. Incubation buckets (Hreiðarsdóttir 2024).

During the application the drying time of the sprayed leaf was noted. The plants needed to get enclosed so the suspension would not dry out. This would be less relevant when whole plants are doused increasing the general humidity for the time being. After 65h of incubation inside the buckets the plants were placed back into their original places, on benches in the greenhouse. The xanthan spray and foam leaf remained with wet surfaces, however the dipped leaves and conventional sprayed leaf had somewhat dried. After a hot day on the greenhouse bench, it was decided to move the plants back into the buckets. For the plants to remain in a more humid cooler environment as seen in figure 6. However, this time the lids were replaced with see-through plastic bags to enable the light to reach them. The buckets remained in the shady part of the greenhouse. The environmental factors during the incubation should have suited the pathogen. Although targeted leaf surface remained wet, the RH was not measured and could have been higher.

All targeted leaves were close to maturity (first set of true leaves in grew in the middle of February) which can delay the visible symptoms. The ontogenic resistance is well recognised with apple scab. Where the young leaf is more susceptible than a mature leaf (Sokolova and Moročko-Bičevska, 2022). The mature leaf will still get infected as previously mentioned but lacks visual symptoms (Li and Xu, 2002). Most studies do not mention the stage of leaves, probably due to whole plants being subjected to the treatments with leaves of varied ages.

6. Conclusion

Apple is one of the most common fruits grown commercially in the temperate regions and the pathogen *V. inaequalis* is one of the more economically damaging diseases to growers (Khajura et al., 2018). As the fungicides, due to their harmful nature, become more strongly regulated. The importance of screening for resistant genes in new cultivars only becomes more adamant. While the experiment resulted in lack of visual symptoms, it sheds light on possible factors that are key to a successful inoculation. Backed up with the literature review findings, the following recommendations were made.

When preparing the inoculum, if the objective is to make a viable count on a petri plate, a fresh batch of green leaves infected with scab lesions should be collected. These leaves will have conidia, which are more likely to grow as a lab culture, as opposed to the ascospores more likely found on the overwintered leaves. If used directly, the conidia could be washed of the leaves. If the pathogen needs to be stored, the leaves should be dried before freezing. The frozen leaves should then be macerated with water and a fine sieve should be used to create a suspension, adjust to around 5 x 10^5 conidia/mL.

It should be ensured that the environment for the pathogenesis remains stable for at least 48 hours with 80 - 100% RH and around 18 - 20°C. This can entail covering the plants, enclosing them, or using a humidifier. It should be kept in mind that a greenhouse warms up quickly on sunny days so it might be necessary to move the plants during the incubation period. Then the application can commence. Due to its ease and wide usage, the spraying method is recommended. The application should focus on young more susceptible leaves and make a note of grading those for the symptoms. High temperatures post-incubation period should also be avoided.

Depending on the screening objective, various grading systems might be useful. To qualitatively grade the symptoms, the most widely used scoring system was developed by Chevalier et al. in 1991. It has fewer classes than other scoring systems which keeps the screening method simple and effective with less space for subjectivity. However, the ECPGR system, apple scab severity and incidence scale, could be considered if more intricate measurements are required (Lateur et al., 2022). None of the grading systems were trialled due to lack of symptoms.

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