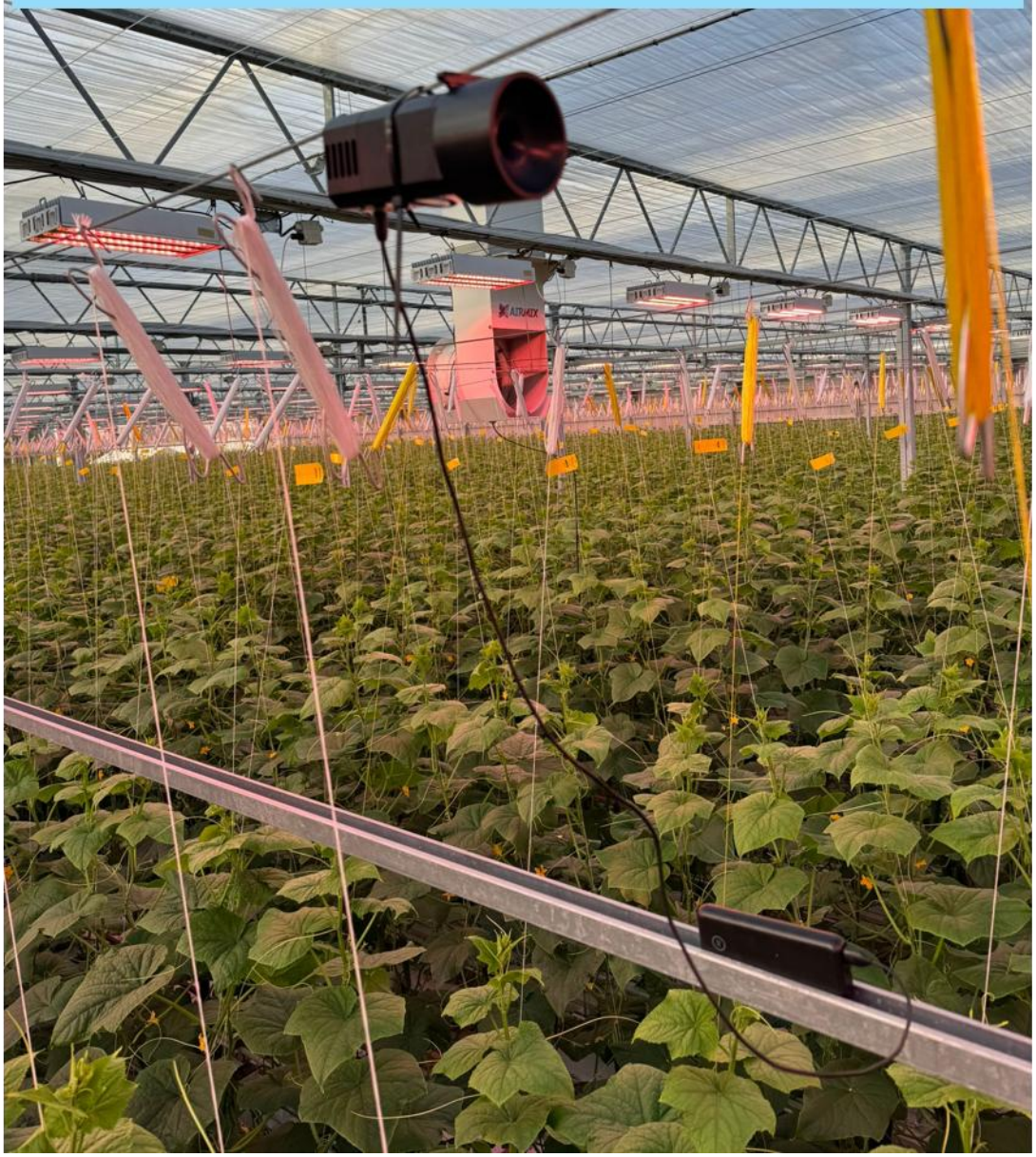
Monitoring of air samples for the presence of plant pathogens within the framework of IPM (LWV21.183)



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Referaat

Verschillende luchtbemonsteringsapparaten zijn getest op hun vermogen om in de lucht overgedragen plant-pathogene bacteriën en schimmels in kassen te vangen gedurende de teelt van komkommers, gerbera's en rozen. Op basis van luchtmonstervolume, efficiëntie om zowel bacteriën als schimmelsporen te vangen, betaalbaarheid, beschikbaarheid en gebruiksgemak, ook bij verwerking van monsters, werden zes verschillende apparaten geselecteerd voor verder onderzoek. Na het optimaliseren van protocollen voor DNA-extractie en detectie van pathogenen met specifieke (qPCR en LAMP) en semi-specifieke technieken (metagenomica), werden de Rotorod (centrifugale impact met roterende arm), Pollensniffer (ventilator-bediende zuiging) en de Coriolis Compact (droge cycloon) allemaal succesvol ingezet in experimentele en/of commerciële kassen om bacteriën (*Pseudomonas amygdalia* pv. *lachrymans*) schimmelsporen van meeldauw (*Podosphaera xanthii* en *P. pannosa*), botrytis (*Botrytis cinerea*) en/of mycosphaerella (*Stagonosporopsis citrulli*) in de lucht op te vangen en te detecteren. DNA van luchtmonsters genomen door de Rotorod en Pollensniffer zijn ook gebruikt in metabarcoding studies, waarbij niet alleen werd aangetoond welke (andere) plantpathogenen aanwezig waren, maar ook een inzicht werd verkregen over welke genera van bacteriën en schimmels algemeen voorkomen en/of specifiek aanwezig zijn in de verschillende kassen.

Abstract

Different air samplers were tested for their ability to capture airborne plant pathogenic bacteria and fungi in greenhouses during cultivation of cucumbers, gerberas and roses. Based on air sampling volumes and the efficiency to capture both bacteria and fungal spores, affordability, availability and ease of use in operation and downstream sample processing, six different devices were selected for further studies. After optimizing protocols for DNA extraction and DNA-based detection of pathogens using targeted (qPCR and LAMP) and semi-targeted approaches (metagenomics), the Rotorod (centrifugal rotating-arm impaction), Pollensniffer (fan-operated suction) and the Coriolis Compact (dry cyclone) were all successfully employed in experimental and/or commercial greenhouses to catch airborne fungal spores of powdery mildew (*Podosphaera xanthii* and *P. pannosa*), botrytis (*Botrytis cinerea*) and/or mycosphaerella (*Stagonosporopsis citrulli*). DNA from air samples collected Rotorod and Pollensniffer air samplers were also used for metabarcoding studies, revealing not only the presence of specific plant pathogens but also providing further insights on which bacterial and fungal genera are specifically and/or omnipresent in the different greenhouse environments.

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Summary

Different air samplers were tested for their ability to capture airborne plant pathogenic bacteria and fungi in greenhouses during cultivation of cucumbers, gerberas and roses. Based on information on air sampling volumes, efficiency to capture both bacteria and fungal spores, affordability, availability and ease of use in operation and down stream sample processing obtained in a literature study, six different devices (MAS-100 Eco, Coriolis Micro, Multi-Vial Cyclone, High-Volume Cyclone, Rotorod and Pollensniffer) were selected for further testing.

After testing and comparing DNA extraction protocols, the MasterPure Yeast DNA Purification Kit was chosen to extract DNA from air samples. Existing and newly developed real-time qPCR and LAMP assays were used for targeted detection of the bacterium *Pseudomonas amygdali* pv. *lachrymans* and the fungal pathogens causing powdery mildew (*Podosphaera xanthii* and *P. pannosa*), botrytis (*Botrytis cinerea*) and/or mycosphaerella (*Stagonosporopsis spp*) in DNA samples. Using qPCR assays targeting multi-copy DNA markers such as ITS, spores could be detected with both devices at a concentration of approximately one spore per m^3. An ultra-fast on-chip qPCR assays developed for detection of *B. cinerea* showed great potential, as results were comparable to qPCR and detection was achieved within 10 minutes. Metabarcoding tools based on Nanopore sequencing of specific markers for bacteria (16S) and fungi (ITS) were also developed and applied to assess the aerobiome in greenhouses.

After using targeted detection approaches (qPCR and LAMP) in experimental greenhouses, the MAS-100 Eco (sieve-based impaction on filters), Rotorod (centrifugal rotating-arm impaction), Pollensniffer (fan-operated suction) and Coriolis Micro (wet cyclone) showed all a high efficacy to catch airborne inoculum from selected plant pathogens. Due to ease of operation and sample processing for DNA extraction, as well as high efficacy to capture both small (bacteria) and larger particles (fungal spores), the Rotorod and Pollensniffer were selected and used to follow the long-term dynamics of botrytis (*B. cinerea*), mycosphaerella (*Stagonosporopsis* spp.) and powdery mildew (*P. pannosa* and *P. xanthii*) in commercial greenhouses during cultivation of cucumbers, gerberas and roses. During the last phase of the project, the Coriolis Compact (dry cyclone) was also successfully used to detect airborne powdery mildew and mycosphaerella spores in a cucumber greenhouse.

DNA from air samples collected by the Rotorod and Pollensniffer were also further used in metabarcoding studies, revealing not only the presence of other specific plant pathogens, such as *Pseudoperonospora cubensis*, the causal agent of cucumber downy mildew, but also further insights on which bacterial and fungal genera are specifically and/or omnipresent in the different greenhouse environments. As expected, There were some differences in the mycobiomes associated with the different greenhouses over time. For example, the highest number of reads in the rose and gerbera greenhouse were measured for *Penicillium* and *Aspergillus* spp., respectively, while high read numbers of *Cladosporium* spp. and *Fusarium* spp. were detected in the cucumber greenhouse. In addition to these genera, high read numbers of *Botrytis* spp. and *Wallemia* spp. were also frequently found in all greenhouses. Further studies using metabarcoding will be needed to check if the different greenhouse mycobiomes, or at least part of it, found in this study are more a reflection of the crop itself or a consequence of the outdoor environment in combination with the indoor environmental conditions of the greenhouse itself.

Future research using air sampling devices, including automated spore trapping devices based on the combined use of imaging methods and artificial intelligence that have recently come onto the market, should focus on crop-disease combinations where spore-trapping data can be used to optimize cultivation conditions or rationalize disease management interventions in the greenhouse. In combination with disease forecasting spore counts can potentially directly guide the use of crop protection products (e.g. timing, choice and rate) as part of an integrated pest management, aiming to maximize control while minimizing environmental impact, human health risks and the development of pesticide resistance. This study should establish how many sampling points are needed in a greenhouse and at which threshold (spores per m^3 air) intervention is needed to mitigate the economic impacts of disease outbreaks. Using DNA-based assays, it is also possible to check if spores are alive or dead and, when markers are available, the presence of important traits like virulence and pesticide resistance can be detected in pathogen populations.

Samenvatting

Verschillende luchtmonsters werden getest op hun vermogen om in de lucht verspreidende plantpathogene bacteriën en schimmels in kassen te vangen tijdens de teelt van komkommers, gerbera's en rozen. Op basis van informatie over luchtmonstervolumes, efficiëntie bij het vangen van zowel bacteriën als schimmelsporen, betaalbaarheid, beschikbaarheid en gebruiksgemak en verwerking van downstream monsters die in een literatuurstudie werden verkregen, werden zes verschillende apparaten (MAS-100 Eco, Coriolis Micro, Multi-Vial Cyclone, High-Volume Cyclone, Rotorod en Pollensniffer) geselecteerd voor verdere tests.

Na het testen en vergelijken van DNA-extractieprotocollen werd de MasterPure Yeast DNA Purification Kit gekozen om DNA uit luchtmonsters te extraheren. Bestaande en nieuw ontwikkelde real-time qPCR en LAMP assays werden gebruikt voor gerichte DNA-detectie van de bacterie *Pseudomonas amygdali* pv. *lachrymans* en de schimmelpathogenen die meeldauw veroorzaken (*Podosphaera xa*nthii en *P. pannosa*), botrytis (*Botrytis cinerea*) en/of mycosphaerella (*Stagonosporopsis* spp). Met qPCR assays die gebruikmaken van multi-copy DNA-markers, zoals ITS, konden sporen in luchtmonsters worden gedetecteerd bij een concentratie van ongeveer één spore per m^3. Een ultrasnelle on-chip qPCR assay ontwikkeld voor de detectie van *B. citeren* toonde groot potentieel, aangezien de resultaten vergelijkbaar waren met die van qPCR en een detectie binnen 10 minuten werd bereikt. Metabarcoding assays gebaseerd op Nanopore sequencing van specifieke markers voor bacteriën (16S) en schimmels (ITS) werden ook ontwikkeld en toegepast om de samenstelling van het aerobioom in kassen te beoordelen.

Door middel van gebruik van gerichte detectiemethoden (qPCR en LAMP) in experimentele kassen toonden de MAS-100 Eco (zeef-gebaseerde impactie op filters), Rotorod (impactie op centrifugale rotatiearm), Pollensniffer (ventilator-bediende zuiging) en Coriolis Micro (natte cycloon) allemaal een hoge effectiviteit om de door de lucht verspreidende inoculum van geselecteerde plantpathogenen op te vangen. Vanwege de eenvoudige werking en monsterverwerking voor DNA-extractie, evenals de hoge effectiviteit om zowel kleine (bacteriën) als grotere deeltjes (schimmelsporen) op te vangen, werden de Rotorod en Pollensniffer geselecteerd en gebruikt om de langetermijndynamiek van botrytis (*B. cinerea*), mycosphaerella (*Stagonosporopsis* spp.) en meeldauw (*P. pannosa* en *P. xanthii*) te volgen in commerciële kassen tijdens de teelt van komkommers, gerbera's en rozen. Tijdens de laatste fase van het project werd de Coriolis Compact (droge cycloon) ook met succes gebruikt om de hoeveelheid door de lucht verspreidende meeldauw en mycosphaerella sporen in een komkommerkas te bepalen.

DNA uit luchtmonsters verzameld door de Rotorod en Pollensniffer werd ook verder gebruikt in metabarcoding studies, waarbij niet alleen de aanwezigheid van andere specifieke plantpathogenen werd aangetoond, zoals *Pseudoperonospora cubensis*, de oorzakelijke factor van komkommer valse meeldauw, maar ook verdere inzichten over welke bacteriële en schimmel soorten aanwezig zijn in de verschillende kassen. Zoals verwacht waren er in de loop der tijd enkele verschillen in de mycobiomen die in verschillende kassen werden gevonden. Zo werden het hoogste aantallen 'reads' in de roos- en gerberakassen gemeten voor respectievelijk *Penicillium* en *Aspergillus* soorten, terwijl 'reads' voor C*ladosporium* en *Fusarium* soorten veel hoger waren in de komkommerkas. Naast deze soorten werden ook relatief hoge aantallen 'reads' voor *Botrytis* en *Wallemia* soorten vaak aangetroffen in alle kassen. Verdere studies met metabarcoding zullen nodig zijn om te controleren of de verschillende kas mycobiomen die in deze studie gevonden zijn, meer een afspiegeling is van het gewas zelf in de kas of een gevolg zijn van de buitenomgeving in combinatie met de omstandigheden binnen in de kas.

Toekomstig onderzoek met luchtmonsters, waaronder geautomatiseerde sporenvangapparaten gebaseerd op de combinatie van beeldvormingsmethoden en kunstmatige intelligentie die recentelijk op de markt zijn gekomen, moet zich richten op combinaties van gewasziekten waarbij sporenvanggegevens kunnen worden gebruikt om teeltomstandigheden te optimaliseren of ziektebeheersingsmaatregelen in de kas te rationaliseren. In combinatie met ziektevoorspelling kunnen sporentellingen mogelijk direct het gebruik van gewasbeschermingsmiddelen (bijv. timing, keuze en snelheid) sturen als onderdeel van een geïntegreerd ziektebeheer, met als doel de maximale controle te bevorderen en tegelijkertijd de milieubelasting, gezondheidsrisico's voor mensen en de ontwikkeling van pesticiden resistentie te minimaliseren. Deze studie moet vaststellen hoeveel bemonsteringspunten nodig zijn in een kas en bij welke drempel (sporen per m^3 lucht) interventie nodig is om de economische impact van ziekte uitbraken te beperken. Met behulp van DNA-gebaseerde assays is het ook mogelijk om te controleren of sporen levend of dood zijn en, als markers daarvoor beschikbaar zijn, kan ook de aanwezigheid van belangrijke eigenschappen zoals virulentie en pesticiden resistentie worden gedetecteerd in populaties van plantpathogenen.

1. Introduction

Background

Importance of greenhouse cultivation for the Netherlands

The greenhouse horticulture sector with approximately 10,000 hectares under glass is very important for the Netherlands and comprises 0.9.% of the gross domestic product with a production value of 6.1 billion euros. Many plants and plant products are grown under glass: pot plants, greenhouse vegetables, soft fruit and cut flowers. The cultivation is optimized regarding sensors for, among other things, carbon dioxide balancing temperature, humidity and lighting in accordance with Het Nieuwe Telen (HNT) concept, that supports the sustainable development of greenhouse horticulture (https://www.glastuinbouwnederland.nl/thema/energie/het-nieuwe-telen/). Several companies use recirculation water monitoring for plant pathogens, but currently no air samples are analyzed for the presence of biological agents such as plant pathogens.

Damage pathogens, role prevention

Globally, an average of 30% of crop yields are lost due to damage from plant pathogens and pests by insects and mites. In greenhouse horticulture with its wide variety of crops, plant pathogens can enter the greenhouse with starting material and spread via recirculation water and air. Starting material is assessed externally but may contain hidden diseases and/or pests. On more farms, the recirculation water is regularly checked for the presence of plant pathogens. Analyzing the air for plant pathogens can additionally contribute to the prevention of disease problems in the greenhouse.

Airborne spread of fungi and oomycetes

Many plant pathogens spread through the air and can infect an entire greenhouse in this way. The first infections in greenhouse crops are often the result of inoculum coming from outside the greenhouse or from the soil or seeds. During cultivation, on the other hand, the main inoculum sources are in the greenhouse itself. Economically important airborne pathogens are powdery mildew, downy mildew and rust fungi, all biotrophic pathogens, which are highly host-specific and rely on living tissue for reproduction and survival. They produce very high numbers of spores that travel through the air. *Botrytis* and *Alternaria* are necrotrophic fungi that spread through the greenhouse air. They cannot infect healthy tissues directly, but dead, dying and senescent cells provide an ideal breeding ground for these types of pathogens. The high numbers of spores produced by biotrophic and necrotrophic plant pathogenic fungi increase the spore pressure in the greenhouse. In addition, there are spores of *Cladosporium*, *Aspergillus* and *Penicillium* which, especially after harvest, can cause rot in the later storage of fruit and flowers.

The success rate of fungal spores to achieve infection is directly related to the concentration of spores in the greenhouse air. The release of spores into the air is determined by climatic conditions in the greenhouse. Spores of *Botrytis cinerea* (Jarvis, 1960) as well as powdery mildew (*Oidium* spp.) (Byrne *et al*., 2000) are mainly released during the day when the relative humidity changes rapidly. Large amounts of mature spores can also be released by mechanical forces such as wind or movements during crop operations (Hausbeck & Pennypacker, 1991). In the case of *Botrytis cinerea*, the concentration of spores in the air can exceed 1000 spores per m^3 of air. After the spores have come loose, the pressure will drop very quickly and after two hours it will again be at a level of 50 spores per m^3 air or lower (Hausbeck & Pennypacker, 1991). Research conducted with spore traps placed at different heights and in which measurement periods of 8 or 16 hours were used, a homogeneous distribution of spores in the greenhouse was found in the air (Kerssies, 1993). This study concluded that rapid transport and distribution of spores take place within a greenhouse. Hausbeck & Pennypacker (1991), on the other hand, used measurement data collected within an hour. The duration of the measurement period therefore determines which dynamics you will observe in the density of the tracks. There are no known measurements in greenhouses for how long the spores of plant pathogens remain in the air or remain alive, or over what distances spores can be transported. Although there are fans in a greenhouse, there is less air circulation in a greenhouse than in outdoor cultivation.

Due to the enclosed nature of greenhouses, airborne fungal spores may accumulate or occur at elevated concentrations, which may be relevant to occupational health (Rodolfi *et al*., 2003; Adhikari, 2016). Exposure to fungi in the greenhouse air of cucumber and tomato crops appeared to be dependent on the plant growth stage and crop handling (Hansen *et al*., 2012). Harvesting and removing old plants provided the highest exposure. Outdoor air is the main source of airborne fungi in greenhouses (Ercilla-Montserrat *et al*., 2017) with increased spore concentrations related to greenhouse ventilation, opening and closing of windows and doors and movement

of people. The spore concentrations also differed depending on the season (Madsen, 2006, Adhikari *et al*., 2011). The most common fungi in the greenhouse air of cucumber and tomato were *Cladosporium*, *Penicillium* and *Aspergillus* followed by *Ustilago*, the rust fungi and *Oidium* (powdery mildew) (Ercilla-Montserrat *et al*., 2017) (Burge *et al*., 1982). *Oidium* provided the highest measured daily trace concentration. After watering, the concentration of spores of these fungi in the air increased significantly (Burge *et al*., 1982). Others also found these fungi in high numbers in the greenhouse air of commercial greenhouses in Spain (Mons, 2004). In addition, *Botrytis* and *Alternaria* were also prominent. *Cladosporium*, *Penicillium* and *Aspergillus* have mainly been found in greenhouses for the propagation of ornamental plants (Li & LaMondia, 2010). This corresponds to measurements in the air of orchids in greenhouses (Magyar *et al*., 2011). First steps have been taken in an air sampling system for powdery mildew spores in rose (*Podosphaera pannosa*) (Leiss & Pham, 2020). A wide range of plant pathogenic fungi and oomycetes can spread through the air. The variety of plant pathogens depends on the crop grown. Main fungal species that can spread through the air are *Botrytis*, *Fusarium*, *Alternaria*, *Mycosphaerella*, *Podosphaera* (powdery mildew) and *Pseudoperonospora* (downy mildew). Depending on the local conditions in the greenhouse, these organisms form conidia and/or ascospores that can infect nearby plants via water splash or through the air.

Airborne spread of bacteria

In greenhouses, plant pathogenic bacteria can be spread on materials such as tools and clothing during cultivation operations (Chang *et al*., 1991). The bacteria can also move from plant to plant via nutrient and drain water (Griesbach & Lattauschke, 1991, Huang & Tu, 2001). Little is known about dissemination by air. Recent research shows that *Pseudomonas amygdali* pv. *lachrymans* (syn. *P. syringae* pv. *lachrymans*), a bacterium that causes leaf spot disease on cucumber, can spread in a polytunnel via aerosols to surrounding plants (Chai *et al*., 2020). In a room where no further cultivation operations were carried out, 12 days after placing diseased plants in the center of the room, a spread of at least 7.5 meters from the source of infection was observed. At that distance, an equivalent of 172 cells per m^3 was measured in a qPCR analysis in a sample obtained via air sampling. Diseased plants were also found at 7.5 meters. Moisture particles containing the bacterium may become detached from the surface of infected leaves when the crop is heated. The particles released from the cucumber plants had a miniscule size of 1.1-4.7 µm and can easily be transported by airflow to neighboring leaves. Stomata of cucumber plants have an opening of 4-5 µm (Wang *et al*., 2012), and thus the inoculum could penetrate the leaf through these natural openings. It can be expected that other plant pathogenic bacteria can also be spread in low densities via aerosols in greenhouses or polytunnels.). Israeli research on *Clavibacter michiganensis* in tomato also found an airborne spread of the pathogen from diseased to neighboring healthy plants (Burdman, Israel, pers. comm.).

Sampling and analyzing new possibilities for airborne inoculum (various approaches)

Greenhouse horticulture in the Netherlands meets strict phytosanitary requirements throughout the entire chain, partly thanks to smart monitoring and detection technology. However, an important gap in the monitoring of plant pathogens in greenhouses is the analysis of air samples. Insects are monitored on sticky traps or with other traps, whether in combination with the use of pheromones or other attractants. Viruses, bacteria and fungi are mainly detected in plant material and/or recirculated water. But recent advances have been made with air sampling systems enabling detection of pollen and smaller particles, including fungal spores and bacteria. At least ten air sampling systems (air samplers) are commercially available that differ in collection capacity and aspiration rate, and the ability to collect live microorganisms, in specificity (the number of different taxonomic groups that are collected), in ease of use and price. There are systems that collect air particles in a liquid, on a solid support, or on a culture medium. This project aims to close this gap by analyzing aerosols for presence of plant pathogens using molecular techniques in a targeted (specific to organism) and a semi-targeted (PCR amplicon sequencing to detect genera of bacteria and fungi) approach.

Project aims

This project aims to develop a method to sample and subsequently analyze air in greenhouses for the presence of plant pathogens (fungi, oomycetes and bacteria). To this end, state-of-the-art techniques in the field of air sampling and (molecular) diagnostics (e.g. qPCR, LAMP, Illumina and Nanopore sequencing) are combined. Data collected on airborne inoculum in relation to instantaneous disease pressure are recorded and will be analyzed together with available metadata (e.g. greenhouse activities, temperature, RH and light. Possibilities of integration of the different components (air sampling, extraction, detection) will be explored.

Expected results

The project is intended to deliver the following products and knowledge:

- Methods combining the use of effective air samplers with fast, targeted and/or amplicon (semi-targeted) sequence analyses for identification and/or detection of key plant pathogens in commercial greenhouses during cultivation of roses, gerberas and cucumbers

- New insights into disease epidemics based on air sampling that can be used to improve cultivation and rationalize early use of crop protection measures based on IPM

- Method for the uniform extraction of DNA from bacterial and fungal spores in collected air samples.

- PCR amplicon sequencing protocols for microbiome analysis

- Optimization and execution of sequence analysis of isolated DNA from bacterial and fungal spores in collected air samples.

- Comparison of qPCR/LAMP assays with sequence analyses.

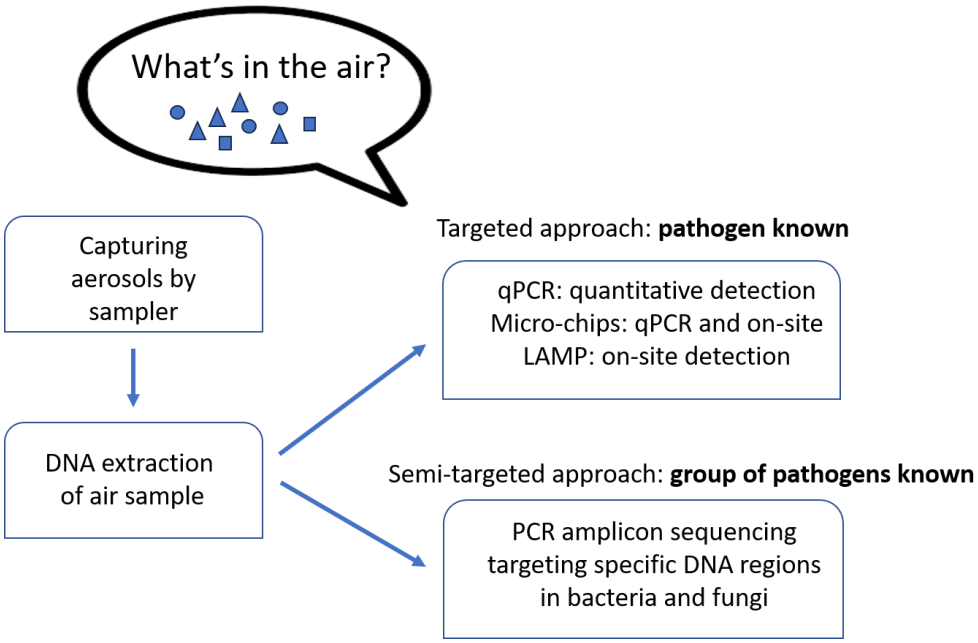
- Sequencing data on 'airborne' pathogens and the relationship with disease outbreaks together with available metadata.

- Training how to use air sampling devices and transfer of knowledge to greenhouse horticultural growers.

- Knowledge transfer through presentations and publications in professional and scientific journals.

- Knowledge transfer to higher education.

- Knowledge from this project can be further rolled out to open field cultivation crops



2. Research approach

The research for the project was carried out in four years according to the initially proposed tasks set at the onset of the project (see Figure 1).

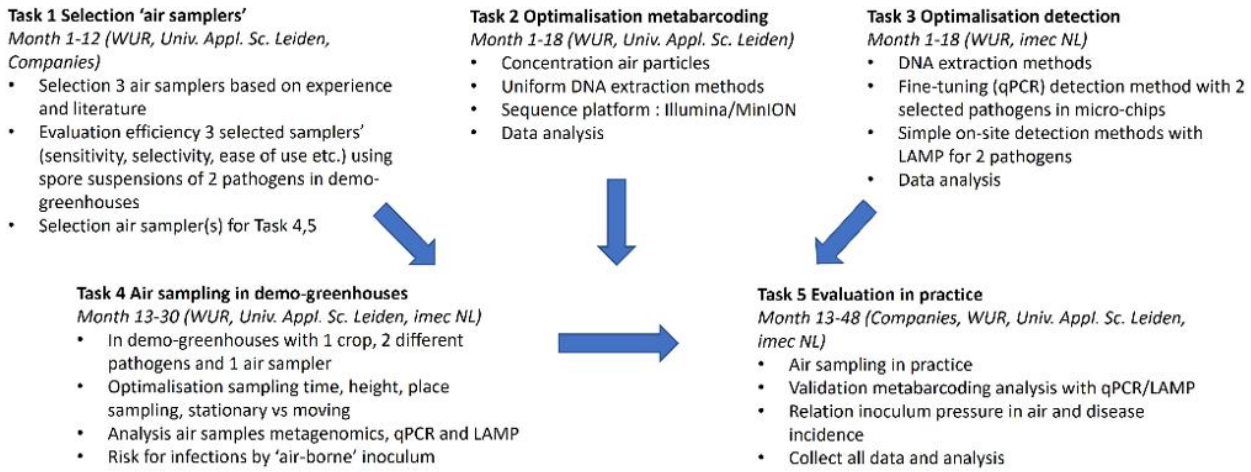


Figure 1. Project workplan

The selected crops and pathogens depend on the choice of the participating Greenhouse Horticultural Cooperatives in the Netherlands. Three Growers Cooperatives will participate: one on vegetables (cucumber) and two on ornamental crops (gerbera and rose). The efficiency of three air samplers targeting at least two airborne pathogens on these three crops were evaluated as part of task 1 of the project. Evaluation was done using DNA extraction protocols and applying molecular detection tools based on targeted (qPCR and LAMP) and semi-targeted detection (metabarcoding) that were developed as part of tasks 2 and 3. After completing tasks 1 to 3, the optimal combination of air sampler with detection tools was used for task 4 (optimal design of sampling and test protocols in demo-greenhouse) and task 5 (applying and validation of tools in practice). The results of the key aspects of the different tasks are further presented as different chapters.

3. Overview of air samplers and target pathogens

3.1 Overview of air samplers

There are different types of active and passive air samplers, and they can be grouped into five categories: wet and dry cyclones, electrostatic impactors, impingers, virtual impactors and filters. For air sampling in glasshouses, we regarded the criteria in Table 1 as needed and/or important.

Table 1. Criteria to select air sampler for catching bacteria and fungal spores in the green house.

air sample flow rate	big volume	IMPORTANT
collection efficiency (particle size)	bacteria and fungi	IMPORTANT
power consumption	220 V and battery	
length of sampling period	long period	
multiple samples	ideally several samples	
ease of processing samples	easy	
active/passive	active needed	
concept sampling	impaction, impinging, filtering, virtual impaction, cyclone (dry, wetwalled and two stage), electrostatic attraction	IMPORTANT
optimal format for analysis	yes	IMPORTANT
maintaining viability	not necessary	
decontamination between samples	cross contamination needs to be avoided	
price of the sampler	for research different then for growers	
autonomeous what period	two kind of autonomous sampling methods	
consumables needed	tubes, plates, filters	

We examined 27 different air samplers that were commercially available at the start of the project (Table 2).

Table 2. Air sampling devices

	Company	**Air sampler**
1	Lanzoni	VPPS 2000 pollen-spore trap
2	MBV	MAS-100 Eco air sampler
3	Bertin Instruments	Coriolis Compact
4	Bertin Instruments	Coriolis Micro
5	AirAnswers	AirAnswers
6	Aerocollect	Aerocollect
7	SKC	Button sampler
8	SKC	Biostage
9	LIS/LCAB	Pollensniffer
10	PCE instruments	Airsampler PCE-AS 1 (PCE).
11	Thermofisher	Aerosolsense
12	Sartorius	MD 8 Air Samplers
13	TCR Tecora	Andersen Cascade Impactor
14	Bioscience	SAS PCR sampler
15	Scannit	Scannit
16	Spornado Sampler	Spornado Sampler
17	Burkard	Multi Vial Cyclone Sampler
18	Burkard	High throughput 'Jet' spore/particle sampler
19	IUL Micro	Spin Air – Air Sampler
20	Agri Samplers Ltd	High-Volume Cyclone Air Sampler
21	Agri Samplers Ltd	DNA auto spore trap
22	Agri Samplers Ltd	LFD auto spore trap
23	Agri isamplers Ltd	Rotorod spore sampler
24	MLU	ChemVol High Volume Cascade Impactor
25	Biomerieux	air IDEAL 3P ®air sampler
26	Maverick PDM	Ionic Spore Trap
27	Research Int.	SASS Air Samplers

These devices differ in collection capacity and aspiration rate, and the ability to collect live microorganisms, in specificity (the number of different taxonomic groups that are collected), in ease of use and price. There are systems that collect air particles in a liquid, on a solid support, or on a culture medium. The last type of 'samplers' is less suitable for an 'untargeted approach' because non-cultivable organisms are excluded. Based on the sampling size (air volume in liters per minute) and the efficiency to capture bacteria and fungal spores (particles size ranging from 1 to 100 µm), availability, affordability and ease of use in operation and down stream sample processing six different devices (see Figure 2) were selected for further studies.

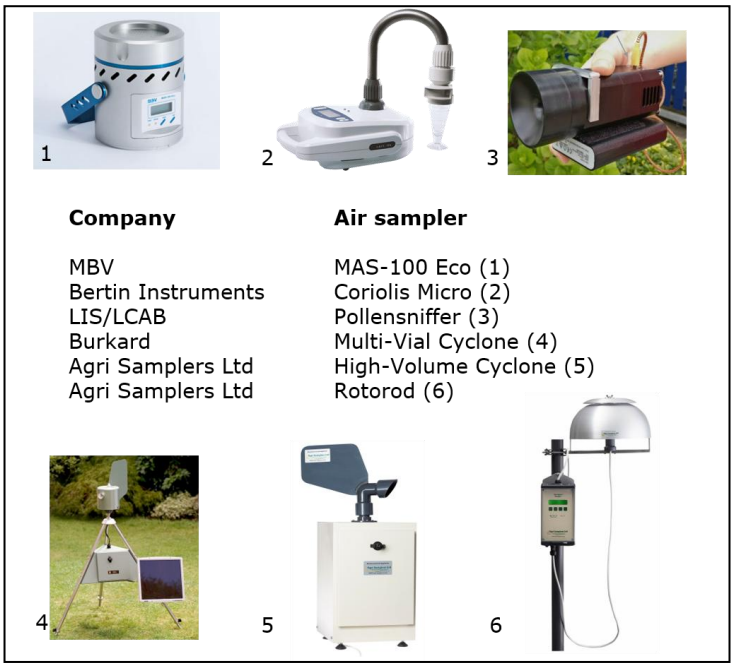


Figure 2. Selection of six air sampling devices for further studies

The six samplers are:

1. MAS-100 ECO (MBV). This air sampler also resembles that of Biostage, but the air flow rate is 100 liters per minute. It collects air particles from 1.6 µm on a nutrient medium. The rechargeable battery works for about 3 hours and can then collect 18 m^3 of air. https://www.mbv.ch/en/microbial-air-samplers/mas-100-eco/
2. Coriolis Micro (Bertin Instruments): The Coriolis Micro air sampler can collect particles from 0.5 to 20 µm from 3000 liters of air in 5-15 ml of liquid in 10 minutes. Catching the particles in a liquid has the advantage that micro-organisms that cannot withstand drying out well (such as bacteria) remain alive, so that vitality can be established. During the collection of the air, liquid evaporates in the tube, so that the sampling time is limited to a maximum (approx. 20 minutes), unless liquid is topped up in between. The system is intended for indoor use but has proven its worth in open field research on the aerosol distribution of Xanthomonas fragariae in a strawberry propagation crop (van der Wolf *et al.*, 2018, Kastelein *et al.*, 2018). https://www.bertin-instruments.com/product/air-samplers/coriolis-micro-air-sampler/
3. Pollensniffer (LIS): At the University of Applied Science Leiden, together with Leiden instrument maker school (LIS), a handy pollen sampler has been developed that captures particles in vaseline (Melinex tape) and which has been validated against a standard volumetric Burkard air sampler (Weger et al. 2020). It's a portable device (diameter 6 cm; length 15 cm; weight 408 g), sampling 8-10 liters of air per minute, and can run 5-6 h from a power bank (205 g). https://www.sciencedirect.com/science/article/pii/S0048969720339267
4. Burkard 'Multi-Vial Cyclone sampler'. The sampler, based on Burkard's miniature cyclone sampler, collects air samples direct into 1.5 ml Eppendorf vials, allowing sample analysis by several techniques including serological (ELISA) and molecular (PCR). Airborne particles can be collected as a dry sample or collected into a liquid as required. The multi-vial sampler contains eight Eppendorf, vials mounted on a Carousel, allowing up to eight sequential samples to be taken. The high efficiency cyclone collects particles less than 1 micron in size with a sample rate of 16.5 liters of air per minute. The Cyclone head is mounted on precision bearings and is connected to a wind-vane that keeps the sampling orifice pointing into the wind. https://burkard.co.uk/product/multi-vial-cyclone-sampler/

5. High-Volume Cyclone (HVC) (Agri Samplers Ltd.). The sampler, sampling 270 liters of air per minute, works by vortexing air into an increasingly narrow vortex, until the air changes direction and exits the system via the center of the vortex, which causes particles to be deposited into removable 2 ml screw-top sample tubes or pots. The HVC has 16 of these sample pots, mounted on a carousel, controlled by a simple-to-operate timer/controller that rotates a new pot into position beneath the cyclone. This allows the user to set up bespoke sampling periods of their choice. The sample pots can be changed individually after each sample is taken, or all together at the end of the specified sampling period for some or all 16 pots. https://agri-samplers.co.uk/product/high-volume-cyclone-sampler-12v-24v-mains/

6. Rotorod air sampler (Agri Samplers Ltd.). The Rotorod spore sampler from Agri Samplers Ltd. has been designed and produced to remotely collect spores, pollen, and other airborne particles autonomously by way of a weatherproof and flexible programmable controller. The unit includes a solar charge controller accepting a max 35W solar panel input to keep the 12V battery fully charged, extending the spore sampling capacity while reducing maintenance time. The rotating arms carrying Vaseline-coated rods (each sampling 37.5 liters of air per minute) rotate at 1700 rpm and a fan fixed to the rotating arm means that new unsampled air is continually being pulled in from below, expelling the sampled air out through the vents in the round metal rain cover. https://agri-samplers.co.uk/product/rotorod-spore-sampler/

3.2 Target pathogens

Greenhouse horticulture in the Netherlands is heavily focused on integrated pest management (IPM), utilizing biological controls for over 90% of cultivated areas. Despite strict hygiene and water treatment protocols, specific fungal, bacterial, and viral pathogens remain primary threats across cucumber, gerbera, and rose crops. Key fungal diseases that these crops have in common are powdery mildews caused by *Podosphaera xanthii* (cucumber and gerbera) and *Podosphaera pannosa* (rose) and gray mold caused by *Botrytis cinerea*, airborne pathogens thriving under high humidity conditions. As a representative for smaller air particles, we also included the airborne cucumber pathogenic bacterium *Pseudomonas amygdali* pv. *lachrymans* (synonym P. *syringae* pv. *lachrymans*), causing angular leaf spot, while the fungal cucumber pathogen *Mycosphaerella cucumis* (synonym *Didymella bryoniae* and *Stagonosporopsis* sp.) causing stem/leaf and fruit rot was also included during the later stage of the project after consultation with growers (Figure 3).

Pathogen	Size (µm)	DNA target sequence
Podosphaera xanthii	15-22 X 24-40	β-tubulin, ITS
Podosphaera pannosa	9.0-17 X 17-29	ITS
Botrytis cinerea	5.0-9.5 X 6.5-12.5	β-tubulin, ITS
Pseudomonas amygdali* pv. *lachrymans	0.5-1.2 X 1.5-3.0	HrpZ
Mycosphaerella cucumis	5.0-8.0 X 15-21	RAPD fragment

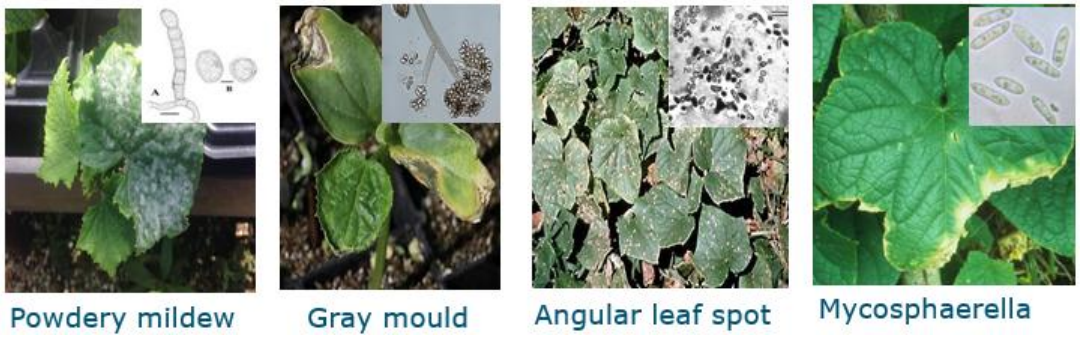


Figure 3. Target pathogens with their size and targeted DNA sequence.

For targeted detection of the pathogens, species-specific sequences, including ITS, Internal Transcribed Spacer, a highly variable DNA region located between the small-subunit (18S) and large sub-unit (28S) ribosomal genes in eukaryotes, β-tubulin, coding protein as part of microtubules, a major component of the eukaryotic cytoskeleton and HrpZ, a bacterial harpin protein, essential for plant infection and shown to able to distinguish *Pseudomonas* spp., were used. The qPCR assays were performed on a QuantStudio 12K Flex Real-Time PCR system (Applied Biosystems).

4. Targeted pathogen detection using qPCR, ultra-fast on-chip qPCR and LAMP

4.1 Primer design

In order to develop a generic DNA extraction method that can be used to compare the efficiency of the different air samplers, we first need to have targeted assays based on qPCR and/or loop-mediated isothermal amplification (LAMP) assays in order to see if all selected pathogens can be detected at low levels after DNA extraction of inoculated samples. After establishing which DNA sequences are available for species-specific detection (see Figure 4), we used the Integrated DNA Technologies (IDT) on-line PrimerQuest tool for design of primers and probes for qPCR (https://www.idtdna.com/) and New England Biolabs LAMP primer design tool (https://lamp.neb.com/#!/) for LAMP assays. The specificity of the sequences for primers and probe was subsequently checked *in silico* by comparing their sequences with available nucleotide sequences in the database using "blastn" program of BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/). For determining the amount of target DNA in samples, we designed short synthetic double-stranded DNA fragments (200-800 bp) containing the target sequence using the IDT gBlocks™ Gene Fragments Entry design software. These gBlocks serve as reliable, stable positive controls for testing and calibration (quantification expressed as number of detected DNA copies). Using the HrpZ gene as marker for specific detection of *Pseudomonas amygdali* pv. *lachrymans* (PAL), the design outputs for the oligos (primers and probe) and the corresponding gBlock sequence are shown in Figure 4. gBlock DNA sequences for the other targets can be found in Appendix 1.



HrpZ Assay Set 8 Details

BACK TO RESULTS

Parameter Set: RT-qPCR (Primers with Probe)
Sequence Name: HrpZ
Amplicon Length: 122

		Start	Stop	Length	Tm	GC%
Forward	CAGTTGATCGGTGAGCTGAT (Sense)	593	613	20	62	50
Probe	ACCTTGGCCTGCAGTCGGTATT (Sense)	615	637	22	68	55
Reverse	TGATCCAGATTCTGTTCGCTAC (AntiSense)	693	715	22	62	45.5

Base	Sequence
1	TAATGGTCAACTAGACGAGAGCTCGCCATTGGGCAAGCTGCTGGCGAAGTCGATGGCGGCCGATGGCATGGCTGGCGGCGGCATTGAGGATGTCATCGCC
101	GCGCTGGACAAGCTGATCCATGAAAAACTGGGTGACAATTTTGGCGCCGCTGCCGACAGTCTGGGCGGTGTCGGACAGCCTGACCTGATGACGCAAGTGT
201	TGAACGGTCTGGCCAAATCGATGCTCGATGATCTTCTGACCCAGCAAGGTAACGGGACTAACTTTTCCAATGACGACATGCCGATGCTGGAAAAGATCGC
301	GCAGTTCATGGATGACAACCCTGCACAGTTTCCTGCGCCGGACTCGGGCTCGTGGGTGAACGAACTCAAAGAAGACAATTTCCTCGATGGCGACGAGACC
401	GCTCAGTTCCGTGCTGCGCTCGATATCATCGGTCAACAGCTTGGCAACCAGCAGAATGATACTGGCGATTTCGCAGCAGGCGATGGCGGCTTGGGCAGCT
501	TTTCAGCCGGTTCATCCGGCCCGGTAGGTGACCCGCTGATCGAAGCCAACACCGGCCCCGGGAACGGTACAAATTCCACGGGTGACGTGGGGCAGTTGAT
601	CGGTGAGCTGATCGACCTTGGCCTGCAGTCGGTATTGTCCGGCGGCGGACTGGGAACCCCCCCGGTCAGTCTGCAGGCAAACGCTTCGCTGGGTAGCGAA
701	CAGAATCTGGATCAGTTGCTCGGCGGCCTGGTGCGTAAAGGGCTGGAAGCGACGCTCATGGGGACTGGTCAGATCGGCAATGACCTGAACAGCAGTGCAG
801	CGCAGGTTGC

TTCGCAGCAGGCGATGGCGGCTTGGGCAGCTTTTCAGCCGGTTCATCCGGCCCGGTAGGTGACCCGCTGATCGAAGCCAACACC

GGCCCCGGGAACGGTACAAATTCCACGGGTGACGTGGGGCAGTTGATCGGTGAGCTGATCGACCTTGGCCTGCAGTCGGTATTG

TCCGGCGGCGGACTGGGAACCCCCCCCGGTCAGTCTGCAGGCAAACGCTTCGCTGGGTAGCGAACAGAATCTGGATCAGTTGCTC

GGCGGCCTGGTGCGTAAAGGGCTGGAAGCGACGCTCATGGGGACTGGTCAGATCGGCAATGACCTGAACAGCAGTGCA

Figure 4. Primers, probe and gBlock based on the HrpZ gene (partial sequence of 810 bp; GenBank accession number HQ171971.2) used for detection of *Pseudomonas amygdali* pv. *lachrymans*. On the top, set of oligos (forward primer, reverse primer and probe) designed by the PrimerQuest tool and below the 330 bp gBlock DNA sequence containing the positions of the primer and probe.

For detection of all fungal pathogens, a similar approach was used and the primer and probe sequences that were designed and used for the qPCR testing are listed in Table 3. For detection of *P. fusca* using the single copy gene B-tubulin as target, a false positive reaction was obtained for the closely-related fungus *P. pannosa*, causing powdery mildew in roses. Therefore, we designed a new assay based on a multi-copy DNA region containing part of ITS1 and the small-subunit (SSU) (18S) rRNA gene sequence.

Table 3. Overview of primer and probe sequences to detect airborne fungal pathogens using qPCR.

Pathogen	Target	Primers (forward & reverse)	Probe[1]	Amplicon size (bp)
Podosphaera xanthii	β-tubulin	F: CATCGACAATGAGGCACTTTATG R: CACCAGACATGACAGCAGAA	ATATGGCGACCTCAATCACCTGGT	104
Podosphaera xanthii	ITS	F: CCACCCGTGTGAACTCTTATC R: CATTTCGCTGCGTTCTTCATC	CGTGCTGTGAGTGTTGTCTGAGGAA	186
Podosphaera pannosa	ITS	F: CACCCGTGTGAACTGAATTTG R: GAGCCAAGAGATCCGTTGTT	CCAACTCGTGCAGTTAGTGCAGTCT	155
Botrytis cinerea	β-tubulin	F: GTTACTTGACATGCTCTGCCATT R: CACGGCTACAGAAAGTTAGTTTCTACAA	ATTTTGGCAGATTGATTACAGGGCAAACTTACA	86
Botrytis cinerea	ITS	F: GTCAGTAATGGCAGGCTCTAA R: GATCCGAGGTCAACCATAGAAA	TACAGGTTCTCGGTGTGCTTCTGC	126
Mycosphaerella cucumis	RAPD fragment	F: GTCCAGAGATGAGGATGGAGT R: GCTTGTAGGCGAATAATGAGCC	CGAAGGATATTGATCTAGACCGCACTTTC	92

[1]Probes were ordered from IDT as double-quenched probes using an internal ZEN quencher. 6-carboxyfluorescein (FAM) at 5' was used as fluorophore and Iowa Black FQ (IBFQ) at 3' as terminal quencher.

4.2. qPCR assays.

The qPCR assays were performed on a QuantStudio 12K Flex PCR system (Applied Biosystems) using the PerfeCTa ToughMix from Quantabio using a standard program (Table 4). For quantitative detection of pathogen DNA, a standard curve for each target was made using a 10-fold dilution series of gBlock, typically containing 1 to 10^6 copies per reaction. When air samples were tested results are expressed as copy number of the target per µl of DNA sample tested. Depending on the aspiration rate of the air sampler and the amount of matrix and sample tested, the amount of pathogen DNA can be calculated per liter or m^3 of air.

Table 4. qPCR reaction mixes and program used on the QuantStudio 12K Flex Real-time PCR system to run the assays.

Quantabio	PerfeCTa Toughmix L-ROX	Rxns 200	€ 245.00
	Component:	volume	Final Concentration
	PerfeCTa Toughmix (2X)	12.5 µL	1X
	Forward primer (5 µM)	1.5 µL	300 nM
	Reverse primer (5 µM)	1.5 µL	300 nM
	Probe FAM (5 µM)	0.5 µL	100 nM
	Nuclease-free water	8 µL	
	DNA template	1 µL	
	Final Volume (µL)	25 µL	
	Quantstudio 12k Flex		
	Run profile:		
	Initial denaturation	95°C; 2 min	
	qPCR cycling	40 cycles	
		Denaturat	95°C; 10 sec
		Annealing	60°C; 30 sec

For quantitative detection of pathogen DNA, a standard curve for each target was made using a 10-fold dilution series of the gBlock sample, typically between 1 and 10^6 copies per µl. Results of the test samples are then expressed as copy number of the target per µl of DNA sample. Results of the qPCR assays using the gBlock calibration curve samples for detection of cucumber powdery mildew (CPM) (*P. xanthii*) and *P. syringae* pv. *lachrymans* (Psl) are shown in Figure 5.

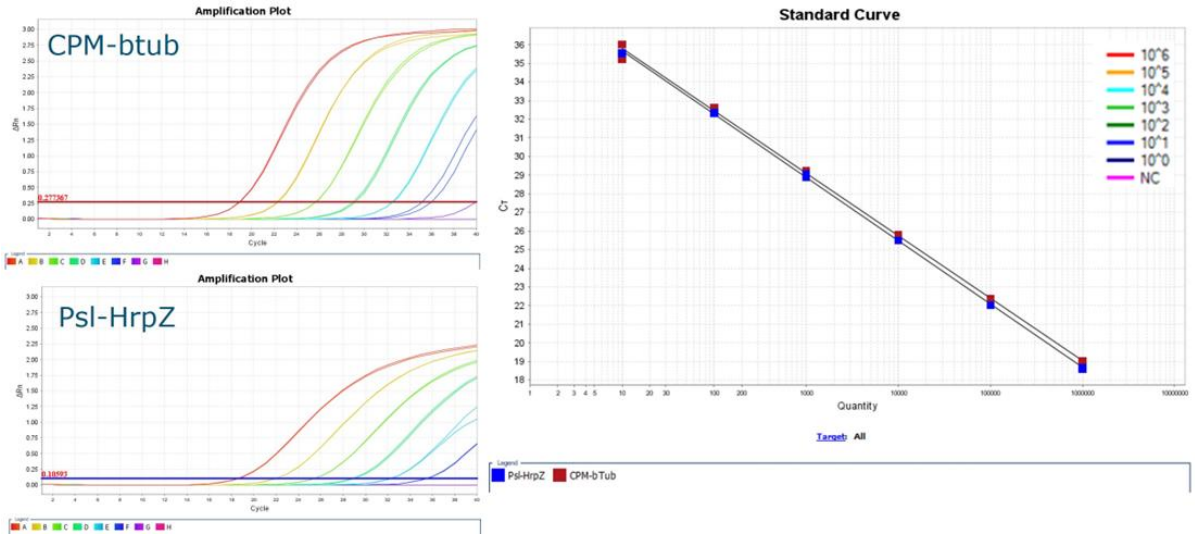


Figure 5. qPCR assays and corresponding calibration curve (PCR detection cycle (Ct) vs. quantity (amount) of target DNA) used for detection of cucumber powdery mildew (*P. fusca*) and *P. syringae* pv. *lachrymans*, using, respectively, β-tubulin and HrpZ as species-specific target sequences.

4.3 LAMP assays.

Published LAMP assays for detection of *P. xanthii/fusca* (Vielba-Fernandez *et al*., 2019), *P. syringae* pv. *lachrymans* (Psl) (Meng *et al*., 2017) and *M. cucumis* (Yao *et al*., 2016) were also evaluated in this study. LAMP assays have been used for rapid and sensitive on-the-spot detection of plant pathogens in the field. It typically employs 4 to 6 primers (F3, B3, FIP, BIP and loop primers LF and LB) targeting 8 distinct binding sites (Figure 6), together with a strand-displacing DNA polymerase to enable amplification under isothermal conditions (Notomi *et al*., 2000).

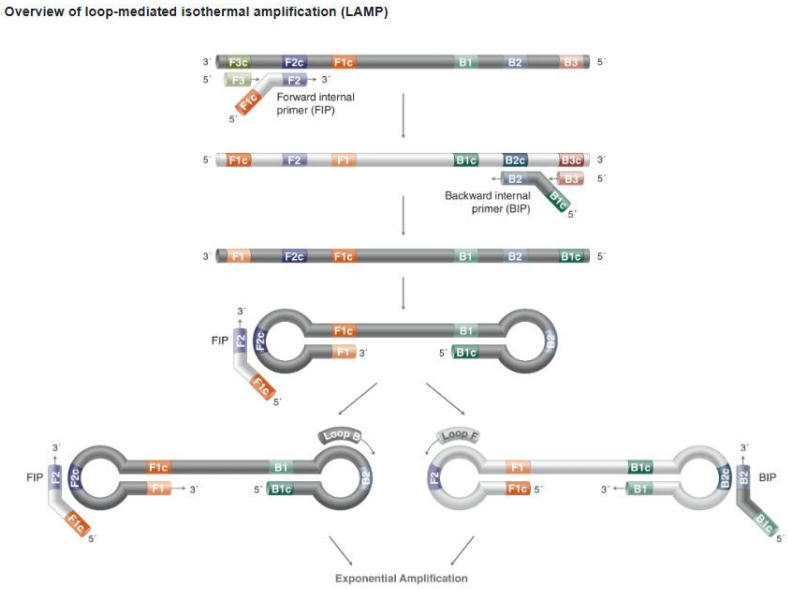


Figure 6. Loop-mediated isothermal amplification using 6 primers (2 loop primers included to facilitate subsequent rounds of amplification) recognizing 8 distinct regions of target DNA (picture obtained from New England Biolabs; https://www.neb.com/).

End-point detection of LAMP products can be done by gel electrophoresis, turbidimetry, color change and with a lateral flow dipstick (LFD), while real-time measuring can be done with DNA-intercalating fluorescent dyes and fluorescent molecular beacon probes. We used the OptiGene Genie II system, a compact portable device, in combination with the ISO-001 master-mix for fast isothermal amplification at 65°C and continuous detection of target DNA using an intercalating fluorescent dye. The reactions were run in a 25 µl volume with end concentrations of each primer from the following primer pairs F3/B3, BIP/FIP and LF/LB at 0.2, 2.0 and 1.0 µM,

respectively. Each reaction consisted of 15 µl master-mix, 8 µl primer mix and 2 µl sample. The following LAMP primers were used for detection of *P. xanthii*, *P. syringe* pv. *lachrymans* and *M. cucumis*:

Detection of *P. xanthii* using β-tubulin as target (Vielba-Fernandez et al., 2019)
BIP 5'-GCGACCTCAATCACCTGGTCTGACCAGGAAACCGTAG-3'
FIP 5'-GAAGGGTTGTTAAGCTTCAATGTCGAAAACTCTGATGA-3'
LB 5'-CTGCTGTCATGTCTGGTGTGACC-3'
LF 5'-CATAAAGTGCCTCATTGTCGATGC-3'
B3 5'-AACTTTCGTAGATCGGAGTT-3'
F3 5'-CTCTGTCCACCAGCTAGT-3'

Detection of *P. syringe* pv. *lachrymans* using HrpZ as target (Meng et al., 2017)
BIP 5'-CCGGTCAGTCTGCAGGCAAAGCCGAGCAACTGATCCAG-3'
FIP 5'-CCAAGGTCGATCAGCTCACCGCGGGAACGGTACAAATTCCA-3'
LB 5'-CGCTGGGTAGCGAACAGAA-3'
LF 5'-ATCAACTGCCCCACGTCAC-3'
B3 5'-CAGCCCTTTACGCACCAG-3'
F3 5'-GCTGATCGAAGCCAACACC-3'

Detection of *M. cucumis* using a sequence-characterized amplified region (RAPD) as target (Yao et al., 2016)
BIP 5'-CCGCATCCGACATCACCCTTGCTTCGCCTTCCTCATCG-3'
FIP 5'-GTGAGGGCCCTGAGATGTTTGAATTATTCGCCTACAAGCCGC-3'
LB 5'-TCCACAAGGTCCCGCAAT-3'
B3 5'-GCGAACTGGCCAATGTGT-3'
F3 5'-AGACCGCACTTTCGAGCT-3'

4.4 Comparison of assay sensitivity: qPCR vs LAMP.

For detection of *P. syringae* pv. *lachrymans* and *P. fusca*, the sensitivity of the LAMP assay was compared with qPCR using dilution series of gBlock samples (copies of target DNA) (Figure 7).

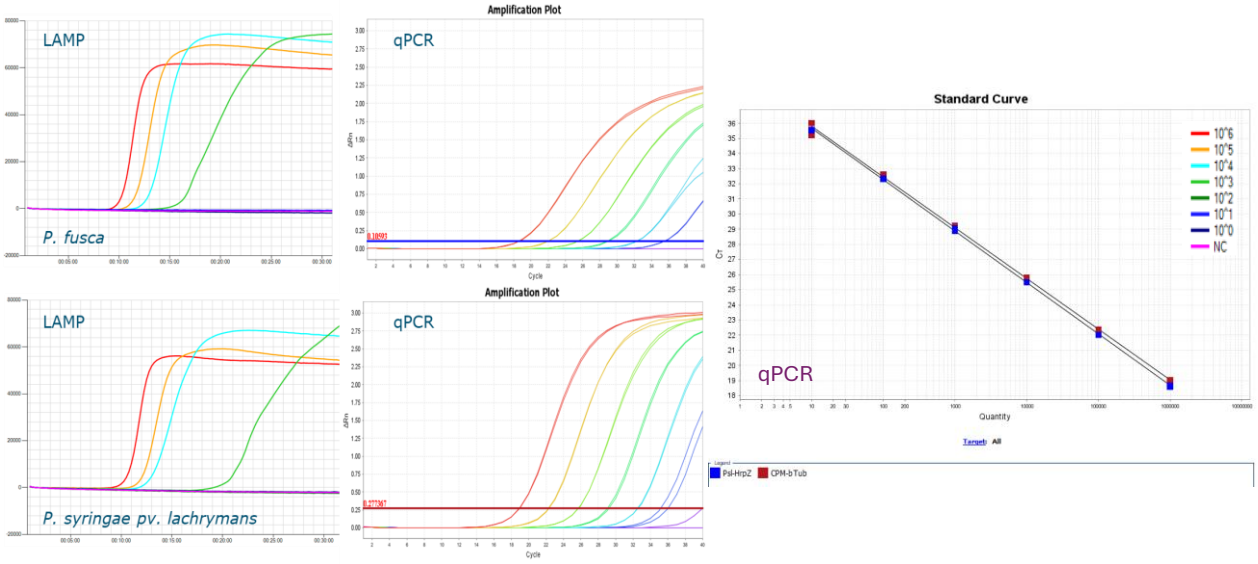


Figure 7. Comparing the sensitivity of LAMP assays and qPCR assays regarding detection of gBlocks DNA samples representing target sequences of *P. fusca* and *P. syringae pv. lachrymans* (testing of dilutions series representing 1 to 10^6 target DNA copies). On the right standard curve relationship between detection time (Ct values) and amount of target copies (gBlocks).

The sensitivity of the qPCR assays was higher than that of the LAMP assays with samples ranging from 10 to 10^6 target DNA copies testing positive while the LAMP assays could only detect samples containing 10^3 to 10^6 copies of target DNA. Due to the higher sensitivity and the wider dynamic range of detection, qPCR assays were further used to detect airborne spores in air samples.

4.5. Development and application ultra-fast on-chip qPCR assays

OnePlanet/IMEC has worked further on the application of an ultra-fast on-chip qPCR platform (Figure 8) for rapid detection of *B. cinerea* using both ITS and β-tubulin DNA sequences as target.

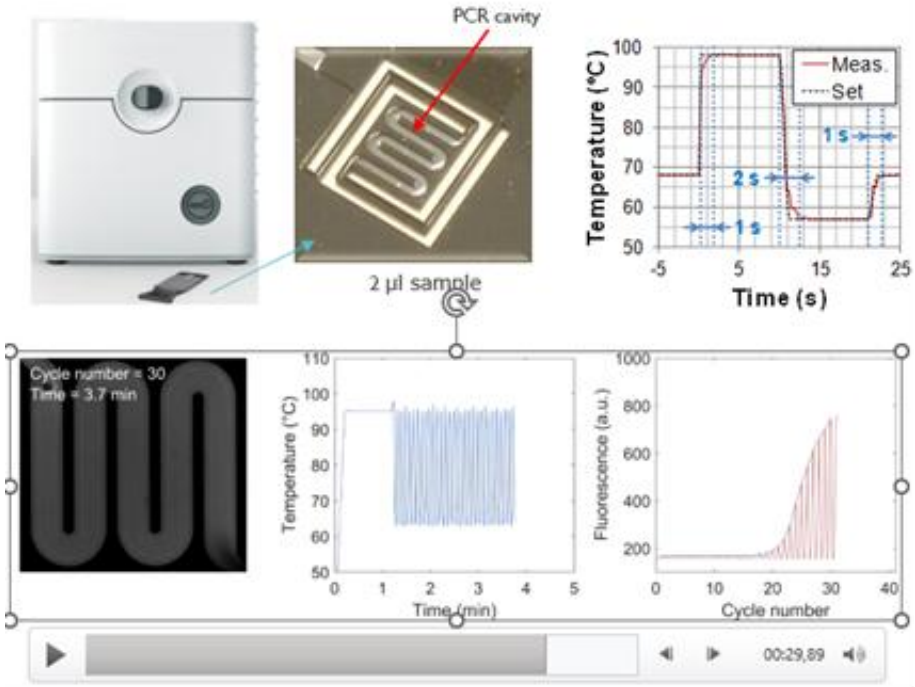


Figure 8. Reader with small footprint reads the PCR card (silicon chip) and performs ultra-fast thermal cycling and optical detection. Silicon chip processes a small sample via a miniaturized network of channels enabling fluidic operations. Fluorescence is measured in real-time due to cleavage of fluorescent labelled DNA probe.

After further optimization of the reagent concentrations, including testing different PCR mixes and establishing optimal concentrations for BSA, primers and probes, as well as cycling conditions, 10 copies per reaction could be reliably detected within 10 minutes using artificial synthesized DNA (gBlocks) (Figure 9).

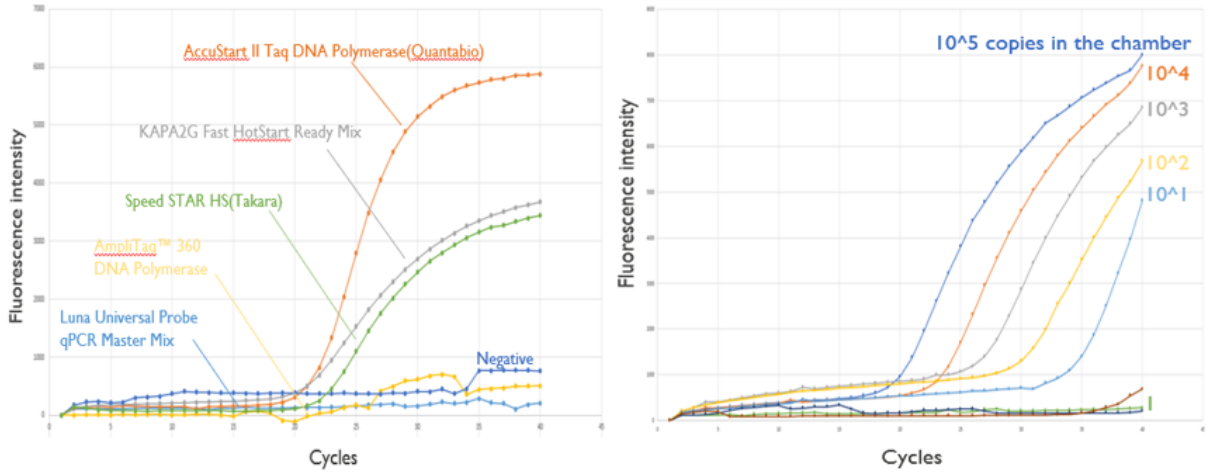


Figure 9. Comparison the sensitivity of different PCR kits during assay development (left) and sensitivity of the ultra-fast qPCR on-chip assay for detection of *Botrytis cinerea* β-tubulin gene copies using the Takara Speed STAR HS PCR kit within 10 minutes after optimization.

The final concentrations of reaction mix reagents in 25 µl using the Speed STAR HS PCR kit (Takara) were: 1X buffer, 1 U Taq Polymerase, 200 nM dNTPs, 0.5 µg ml^{-1} BSA, 1.2 µM of both forward and reverse primer and 400 nM of FAM labelled probe. One µl of DNA sample was added to 17 µl of reaction mix and 1.8 µl of this mix tested in the chip. The reaction conditions were 15 seconds at 95°C (initial denaturation), followed by 40 cycles of 1 sec at 95°C and 3 sec at 60°C with a 5°C s^{-1} second ramp rate.

4.6. On-site LAMP assays for detection of *Mycosphaerella cucumis* in cucumber tissues

Although LAMP assays were not used further to screen air samples for the presence of fungal pathogens, the method is highly suited for on-site confirmation of disease symptoms. Due to the focus on *M. cucumis* in the later stage of the project, we investigated if the published LAMP assay published by Yao *et al*. (2016) could be used on-site to detect the fungus in infected fruits according to our protocol within one hour (Figure 10).

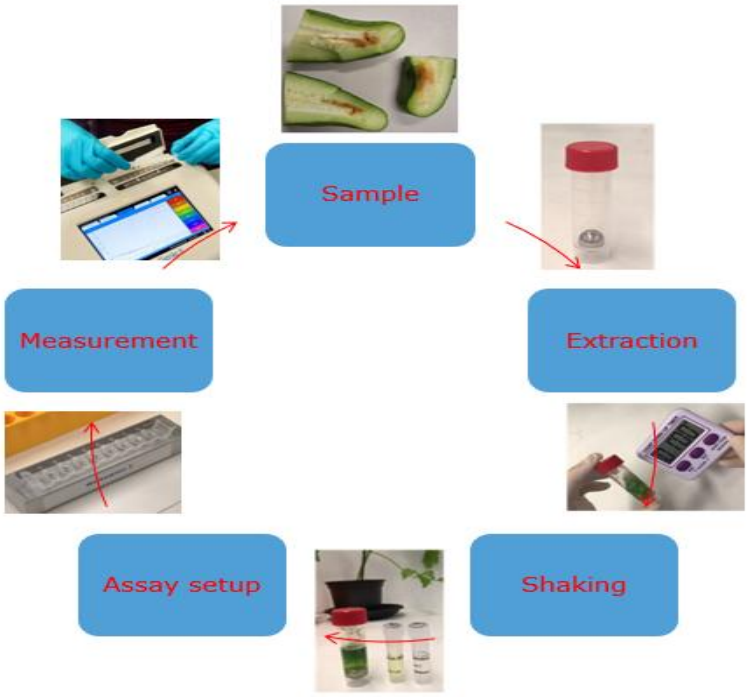


Figure 10. On-site detection of mycosphaerella-infected cucumber fruits.

The extraction step of the protocol, manual shaking of tube, mixing sample with buffer solution and steel ball, takes currently 20 minutes but the presence of mycosphaerella in fruits with symptoms was confirmed within 10 minutes of the 30 minutes lasting LAMP assay. Other fungi such as *Botrytis cinerea* tested negative but this assay cannot distinguish between *Stagonosporopsis cucurbitacearum* and *S. citrulli*, the two closely related species which names are currently being used as the causal agents for gummy stem blight and black rot in cucurbit crops, including watermelon, melon, cucumber and squash, thereby replacing previously used names for this disease such as *M. cucumis*, *D. bryoniae* and *Phoma cucurbitacearum*. Further research should focus on the detection of both species in both asymptomatic and symptomatic fruits and flowers.

5. DNA extraction and selection of air samples for further studies on detection of pathogens in greenhouse experiments

5.1. Air samplers and sampling matrices

The six selected air samplers have different matrices to capture the air particles and to extract the DNA from (Figure 11). For the Coriolis Micro a liquid sample needs further processing, a filter comes from the MAS-100, two plastic rods coated with Vaseline are representative for the Rotorod while dry pellets in small tubes are available from both cyclone air samplers.



Figure 11. Air samplers with their corresponding sampling matrices. From left to right: Coriolis Micro, Pollensniffer, MAS-100, Multi-Vial Cyclone, High-Volume Cyclone and Rotorod air sampler.

5.2. Testing of DNA extraction methods

The ideal criteria for a DNA extraction method are high yields for all target organisms, simplicity (low labor) and low costs while the resulting DNA can be used for different applications (e.g., qPCR and metagenomics), so preferably high molecular weight DNA without presence of inhibitors for DNA amplification. A range of DNA extraction kits with modifications were tested (not all shown), including Echolution, Promega Wizard Food Kit, Promega Wizard Genomic and Qiagen water (Figure 12).

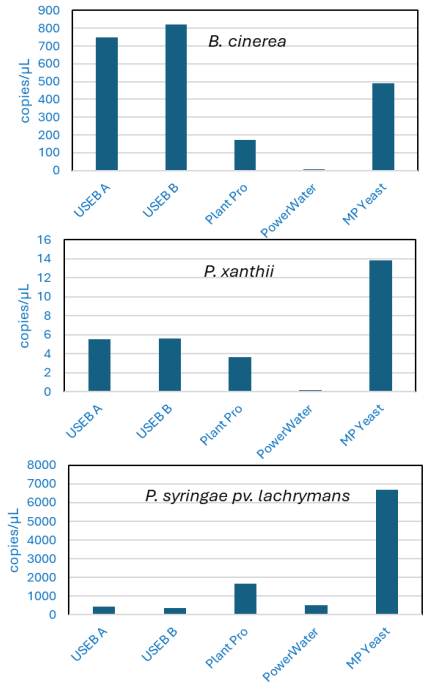


Figure 12. Testing efficacy of different DNA extraction kits (USEB (home-made kit), Qiagen DNeasy Plant Pro, DNeasy PowerWater and MasterPure Yeast DNA Purification (MPY)). Matrices were spiked with equal amounts of spores of each pathogen; target DNA was extracted and detected and quantified using qPCR assays.

After testing spore suspensions from B. *cinerea*, *P.* xanthii and *P. syringae* pv. *lachrymans*, the MasterPure Yeast DNA Purification (MPY) kit was selected due to its high efficacy of recovering DNA from all three pathogens in comparison with the other kits. It was also possible to use this DNA extraction method on the different sampling matrices. The final protocol based on the MPY kit can be found in Appendix 2.

5.3. Greenhouse pilot inoculation experiment to evaluate the easy use and efficacy of different air samplers to capture bacterial and fungal aerosol populations

To compare the six samplers, an experiment was designed to operate the six different air samplers at different positions at similar heights in the greenhouse and rotate these after each inoculation (Figure 13). The inoculation was carried out with a mixed spore concentration of all three target pathogens (each at 10^6 spores m^{-3} of air) using a nebulizer at a height of 3 meters in the greenhouse (Figure 13).



TABLE 1
Position 1
Position 2
Pollen S A (3)

TABLE 2
Position 4
Pollen S B (5)
Position 6

TABLE 2
Pollen S C (7)
Position 8
Position 9

Figure 13. Greenhouse inoculation experiment with different positions of the air samplers

The inoculation was carried out in two experiments with three repeats whereby the three Pollensniffers kept their position and the remaining air samplers were changing from position each time so that every position was taken once. After air inoculation and 10 minutes settling period, samplers were running for 10 minutes, each device sampling a different air volume, depending on their aspiration rate. DNA extractions were carried out and presence of each pathogen measured using qPCR targeting β-tubulin (*P. xanthii* and *B. cinerea*) and *HrpZ* (Psl) (Figure 14).

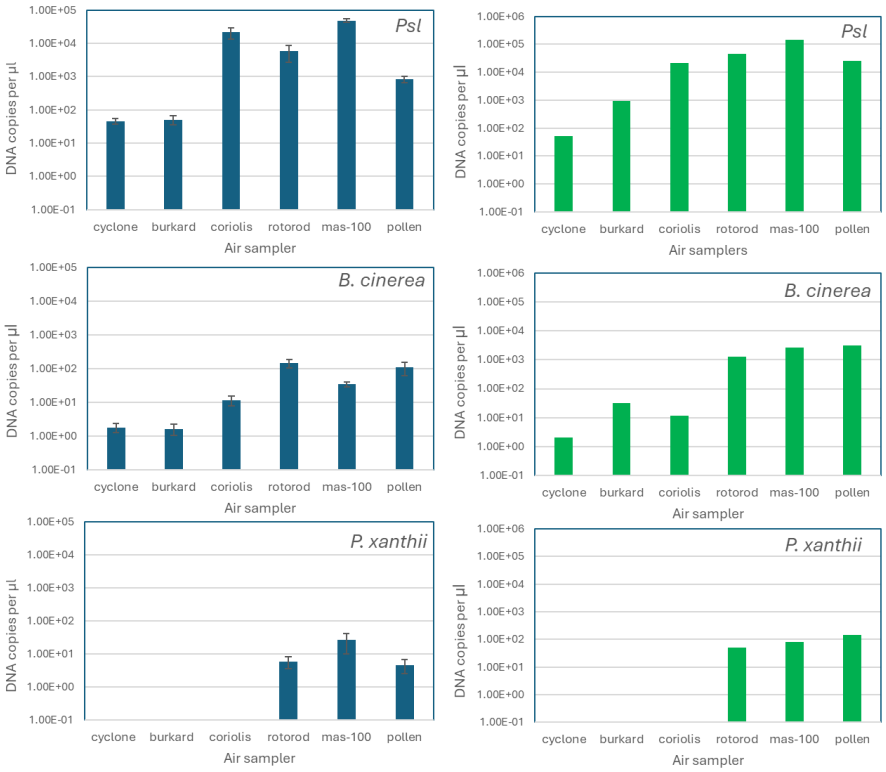


Figure 14. Pathogen detection in greenhouse aerosols collected by different air samplers in 10 minutes. Results expressed as average target-DNA copies in one µl DNA sample (total DNA extract was 30 µl). Left: results from high-volume cyclone (2700 L of air sampled in 10 minutes), Burkard cyclone (160 L), Coriolis (3000 L), Rotorod (375 L), MAS-100 (1000 L) and Pollensniffer (100 L); right, graphs corrected for an equal sample volume of 3000 L.

The MAS-100, Rotorod and Pollensniffer air samplers were highly effective in sampling all three pathogens from the air as shown by the results of the qPCR assays. The Coriolis Micro sampler was also efficient in sampling the smallest particles, represented by the bacterium *P. syringae* pv. *lachrymans* but it was less efficient in sampling the larger fungal spores of *B. cinerea*, with no detection of *P. xanthii*, representing the largest particles (see Figure 4). In comparison with the other air samplers, the cyclones were least effective in sampling both bacteria and fungal spores from the air. The DNA extraction of MAS-100 filters was most laborious and, due to the size of the filter, only a small part of filter was processed for DNA extraction. Therefore, the Pollensniffer and Rotorod air samplers were selected for further sampling of airborne fungal spores in experimental and commercial greenhouses. Both devices can also sample aerosols for 24 hours if needed.

5.4. Demo-greenhouse experiments to evaluate sampling strategies for detection of airborne fungal spores

Several experiments were carried out to establish what sampling protocol is needed to measure the spread of fungal spores and disease transmission in the green house. For the first experiment, which was carried out twice, we inoculated a single cucumber plant with cucumber powdery mildew (*P. xanthii*) which was surrounded by 10 other plants and compared the airborne inoculum with a second experiment where all 11 plants were inoculated with powdery mildew (Figure 15, left). To capture the spores, three Rotorod air sampling devices were placed at three different positions: one opposite to the plants in the corner in orange box (A), one next to the plants in orange box (position B) and one at a height of 1.5 meters above the table next to the plants in red box (C) (Figure 15, right). The Rotorod devices sampled air for 24 hours which is equivalent to 54 m^3 of air for a single rod (flow rate 37.5 L of air per minute). Thirty-five µl of DNA extract was obtained for each daily sample.



Figure 15. Cucumber greenhouse experiment to measure the spread of powdery mildew spores. Spores were inoculated on the plant in the center or on all plants. During incubation, conidia were captured with three Rotorod air sampling devices placed at three different positions in the greenhouse.

Detection of *P. xanthii* target DNA was achieved using qPCR targeting ITS (see Table 3) (Figure 16). With 35 µl of extract available, 54 m^3 of air sampled per rod and assuming 100 ITS copies are present in a single spore, the values in Figure 16 need to multiply by 0.0065 to get an estimation of the number of spores per m^3 air. Up to 6 days after inoculation the highest number of spores were generally detected near the plants at 1.5 meters height. After this period the highest number of spores were detected next to the plant with spores exceeding 1 spore per m^3 after 8 and 11 days in the single plant inoculation experiments and after 7 days in the inoculation of all plants. It is likely that these spores have been produced from new pustules as powdery mildew is known to have a short latency period of 7-14 days depending on environmental conditions. It is clear that spores of P. *xanthii* are not homogeneously distributed in the greenhouse with the lowest number of spores detected furthest away from the inoculum sources opposite of the inoculated plant(s) in the corner of the greenhouse. At this position, only twice a spore concentration higher than one spore per m^3 of air was detected during the three experiments.

To see how far *P. xanthii* spores can be collected from the inoculum source a second experiment was designed and carried out twice. For this, we used three sampling positions, each at two different heights, and air sampling was carried out simultaneously with six Pollensniffers. The positions were next to the plants at 50 cm distance, on the middle table at 7 meters distance and on the edge of the greenhouse at 13.5 meters distance. Pollensniffers were positioned at table level and at a height of 1.5 meters at each table position (Figure 17). To validate the results, a leaf bioassay experiment on a 3 by 3 grid in the greenhouse was also carried out in the second trial. For this assay, leaves with cucumber leaves on petri-dishes were also daily used to catch spores and further incubated in vitro.

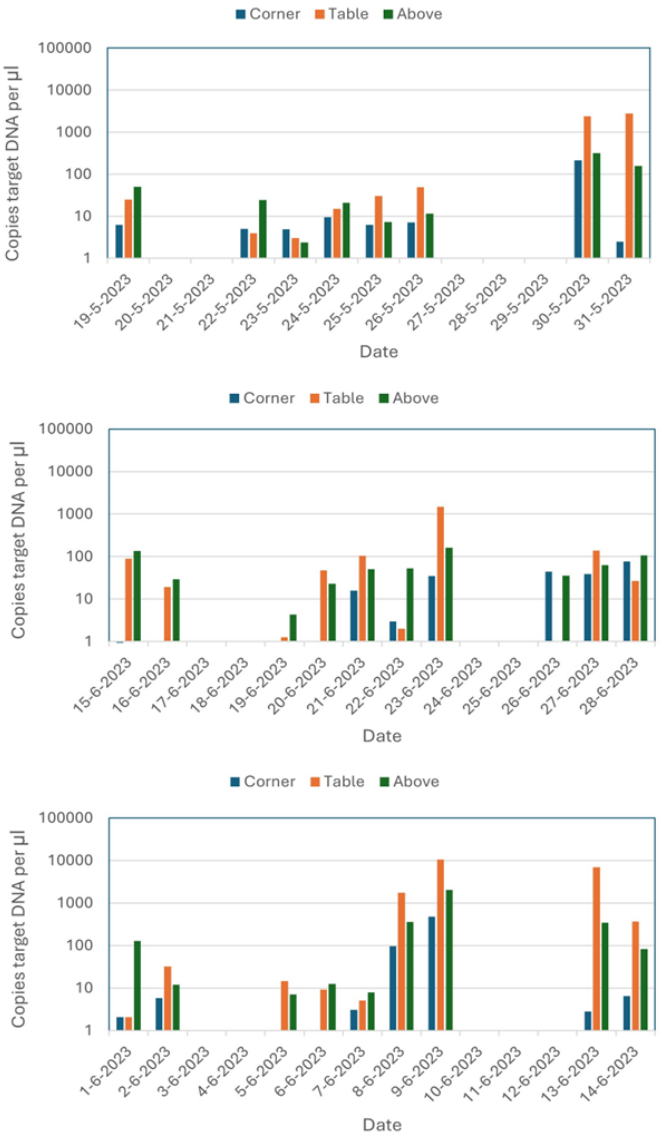


Figure 16. Detection of *P. xanthii* in greenhouse aerosols after inoculation of a single plant in the center (top two displays) or after inoculation of all plants (bottom display). Results expressed in daily copies of target DNA per µl DNA extract as measured by qPCR using ITS as target (average values of two measurements are shown). Approximately 150 DNA copies correspond to one spore per m^3 of air. Days without any results were not sampled.

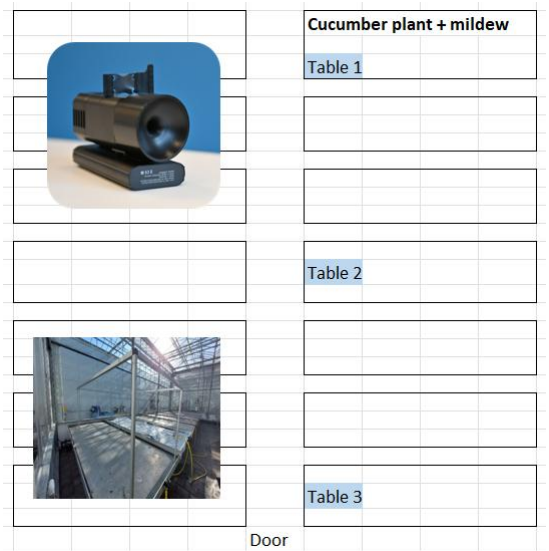


Figure 17. Design of second single plant inoculation experiment in cucumber greenhouse (14 X 9.3 meters).

The second single plant inoculation experiment was also carried out twice. Single plants were infected with 18 mL mildew spore suspension (10^5-10^6 spores per ml), and air samples were collected at three positions and two heights for a duration of up to 3 weeks using Pollensniffers. The results are shown in Table 5.

Table 5. Results expressed as daily DNA copies of target DNA per m^3 from two different trials measuring powdery mildew airborne spore dispersal after inoculation of a single plant in the cucumber greenhouse. Average values of two measurements using qPCR targeting ITS are presented. One spore contains approximately 100 ITS copies.

Sampling date	3-03-24	4-03-24	5-03-24	6-03-24	7-03-24	8-03-24	11-03-24	12-03-24	13-03-24	14-03-24	15-03-24	18-03-24	19-03-24	20-03-24	21-03-24
Day	day 1	day 2	day 3	day 4	day 5	day 6	days 7-9	day 10	day 11	day 12	day 13	days 14-16	day 17	day 18	day 19
T1, next to plant	1.0	0	0.4	0	2.4	93.1	28.7	38	5.9	1.5	61.8	0.4	6	113	104.9
T1, 1.5 m height	3.2	4.4	1.3	25.7	36.7	3.3	38.7	116.9	2.5	18.7	254.4	221.3	808.1	2680.9	15.4
T2, middle	11.4	3.3	20.7	2.2	0	6.1	5.7	54.2	7.7	0.4	1.2	0.8	411.4	235	0
T2, 1.5 m height	2.9	9	0	4.8	2.2	2.2	5.3	26.5	3.2	89.3	74	1.8	252.7	85.7	32.7
T3, edge	0.0	0.8	1	18.9	1	17.1	2.7	5.1	9.9	0.2	44.6	7.9	1.8	299.9	21.5
T3, 1.5 m height	6.8	0.3	0	2	2.2	25	165.2	13.2	0.7	28.9	2.5	2.9	35.4	107.9	82.6

Sampling date	30-09-24	1-10-24	2-10-24	3-10-24	4-10-24	7-10-24	8-10-24	9-10-24	10-10-24	11-10-24	14-10-24	15-10-24	16-10-24	17-10-24	18-10-24
Day	day 1	day 2	day 3	day 4	day 5	days 6-8	day 9	day 10	day 11	day 12	days 13-15	day 16	day 17	day 18	day 19
T1, next to plant	56.3	245.6	33.6	83.5	43.1	39.2	30.8	59.2	175.5	55.1	397.2	42.4	151.6	735	1274
T1, 1.5 m height	69.2	177.2	17.3	98.6	141.6	29.5	92.4	109.9	142.9	467.2	180.3		184.2	1436	52.8
T2, middle	72.8	79	21.5	47.5	44.5	11.3	41.6	48.2	68.1	167.9	18.1	146.6	31.7	145.7	96.6
T2, 1.5 m height	73.5	213.1	20.5	104.8	35	16.3	94.5	44.2	84.2	19.7	58.1	69	23.8	38.9	366.1
T3, edge	84.4	239.8	32.7	47.4	142.1	52.1	290.7	114.2	78.5	10.8	8.4	15.3	53.4		380.3
T3, 1.5 m height	57.9	119.4	23.6	14.5	21.5	14		127	85.1	39	27.6	69	116.7	41.3	259.9

The results of the two trials show a similar trend. Although initially more spores were detected in the second greenhouse trial after inoculation, due to higher inoculum level and/or background levels of airborne spores of *P. xanthii*, the first increase in spore concentration was measured after 9-10 days, followed by a second wave reaching maximum levels of spores after 18 days. These waves are most likely caused by the latency period of powdery mildew, developing new pustules and releasing spores every 9 days. The progress of disease symptoms in the second trial is shown in Figure 18.

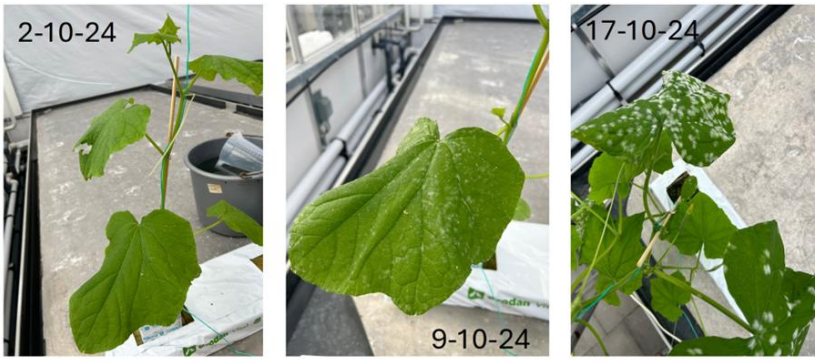


Figure 18. Cucumber powdery mildew disease development after inoculation in the greenhouse on 30 September 2024. Two days (2/10/24), 10 days (9/10/24) and 18 days (17/10/24) after inoculation.

The first visible spots were visible on inoculated leaves after 10 days in both trials, while multiple leaves with sporulating pustules were seen higher in the canopy after 18 days. As expected, the highest airborne levels of spores were detected by the Pollensniffer positioned on table 1 (T1) near the inoculated plant, at both table height and at 1.5 meters height. Lower concentrations of spores were occasionally, but more often detected on the middle table T2 and the T3 table positioned at the edge of the greenhouse towards the end of the experiment. Due to leaf quality issues, the leaf bioassays didn't work well but it showed that live spores could spread at least 14 m away from the plant. Destructive measurements on the last day of the experiment on 18/10/24 showed that the plant length was 2.03 meters higher than the 1.5 m heigh sampling point and $2.6*10^7$ spores were harvested from the plant confirming its potential for spore dispersal.

The experiments showed that both types of air samplers need to be positioned close to diseased plants. The use of multiple spore traps in combination with a rapid detection method can provide an early warning system for disease development in the greenhouse and guide timely use of plant protection measures. Another option is the use of air samplers that can move and sample throughout the greenhouse. For example, air samplers can be positioned on harvesting cars, and the use of drones can be considered as well.

5.5. Timing of air sampling and detection of pathogens in a commercial greenhouse

In order to find out if there are differences in the release of airborne spores of pathogens during a 24 h period, one experiment was conducted in a rose greenhouse studying the dispersal of mildew (*P. pannosa*) and botrytis (*B. cinerea*) spores during the afternoon (13:30-21:30), night (21:30-05:30) and the morning (05:30-13:30) using multiple Pollensniffers for three consecutive days (Figure 19).

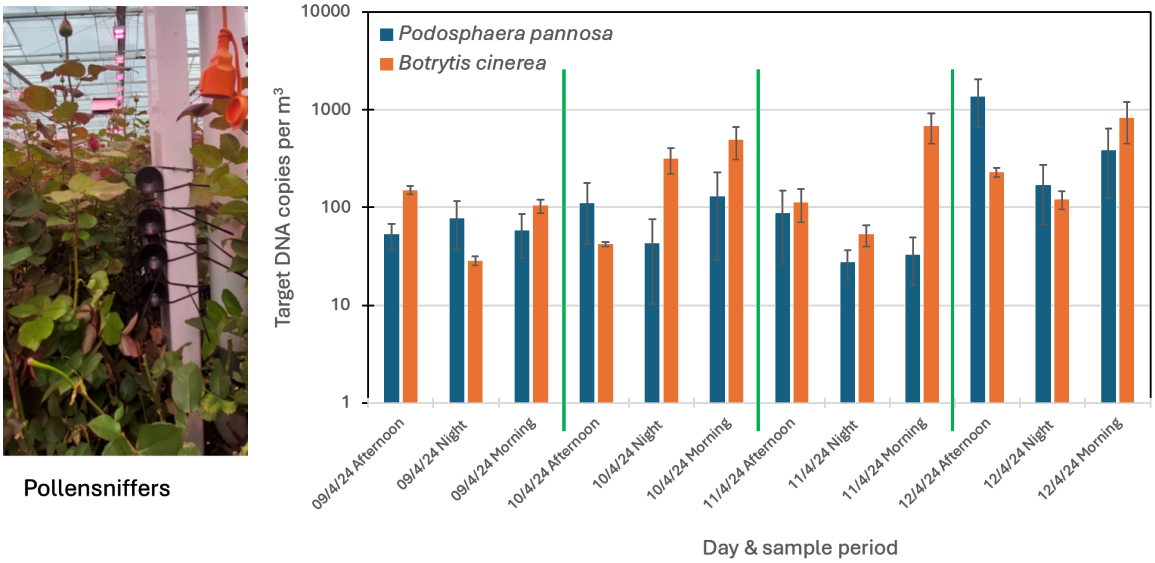


Figure 19. Air sampling and detection of mildew and botrytis spores in a commercial rose greenhouse using Pollensniffers and qPCR. For detection of botrytis and mildew, beta-tubulin (single copy) and ITS sequences (multiple copies), respectively, were targeted.

Considering a 100-fold lower detection sensitivity, botrytis spores were detected at much higher concentrations in the air than powdery mildew spores. For both pathogens, the lowest number of spores were detected at night. The highest levels of botrytis spores were detected in the morning, while mildew spores were best detected in the afternoon.

5.6. Air sampling and detection of pathogens on a harvest car and a drone in greenhouses

The use of air samplers on a drone and in a moving vehicle was also explored. A drone with Vaseline-coated rods of a Rotorod sampler attached was flown for a few minutes in and outside of an anthurium greenhouse (Figure 20). Samples of this drone were analyzed with metagenomics (see chapter 7 for results).



Figure 20. Drone flight with rods sampling aerosols in an anthurium greenhouse. Rods are indicated with red arrows.

In addition to drone flights, aerosols were also sampled with a Pollensniffer positioned on a harvesting car in a rose greenhouse. Air samples (150 L) were taken every 15 minutes when the harvesting car was moving slowly through two rows (one row without plants showing powdery mildew (row C) and one row with plants showing powdery mildew symptoms (row M)). Results of the samples (5 samples per row were collected), regarding detection of botrytis and powdery mildew spores in sampled aerosols using qPCR targeting ITS of these two fungi (*B. cinerea* and *P. pannosa*), are shown in Figure 21.

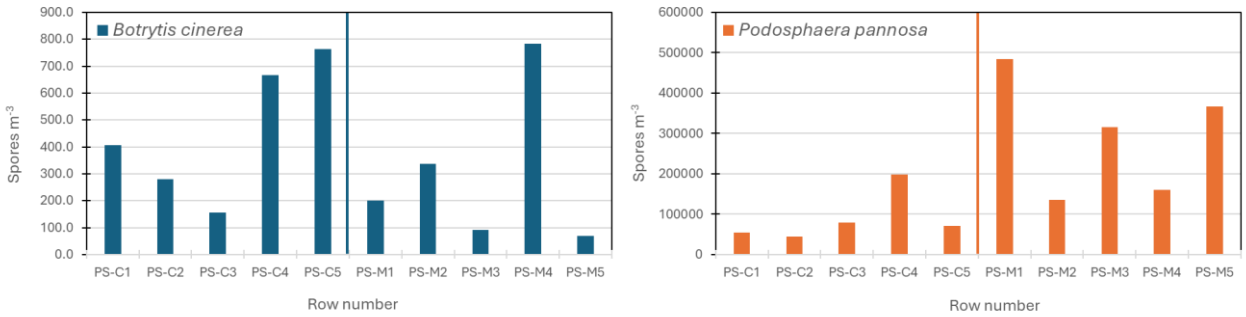


Figure 21. Detection of airborne botrytis and powdery spores on a harvest car in a commercial rose greenhouse. Air samples were collected with a Pollensniffer and pathogens detected using qPCR assays targeting ITS. Results are expressed as spores m^{-3} assuming 100 ITS copies are present in a single spore. On the left of each figure 5 samples from row C; on the right 5 samples from row M.

As expected, due to the presence of powdery mildew in row M, high levels of *P. pannosa* spores were measured in the greenhouse. In comparison with row C, higher spore concentrations of *P. pannosa* were measured in row M where powdery mildew infected plants can be seen. The levels of botrytis spores in aerosols were much lower in both rows and there was not much difference between the rows.

6. Targeted detection of fungal pathogens in aerosols sampled from commercial gerbera, rose and cucumber greenhouses

6.1. Pilot experiment with growers to operate air samplers for detection of botrytis and powdery mildew in commercial greenhouses

After evaluating the different spore trap devices, the Pollensniffer and Rotorod air samplers were further tested by six growers for their potential of application. Instructions were given to the growers on how to collect 24 h samples in two cucumber, two gerbera and two rose commercial greenhouses for one week (Figure 22). Samples were frozen until they were processed for DNA extraction and tested for presence of pathogens using qPCR.



Figure 22. Operation of the Pollensniffer and Rotorod air sampler in a cucumber, gerbera and rose greenhouse

The results from the samples collected by the two air samplers were very similar. Clear qPCR amplification curves were obtained from all greenhouses (see example in Figure 23), indicating that both pathogens were present in most samples.

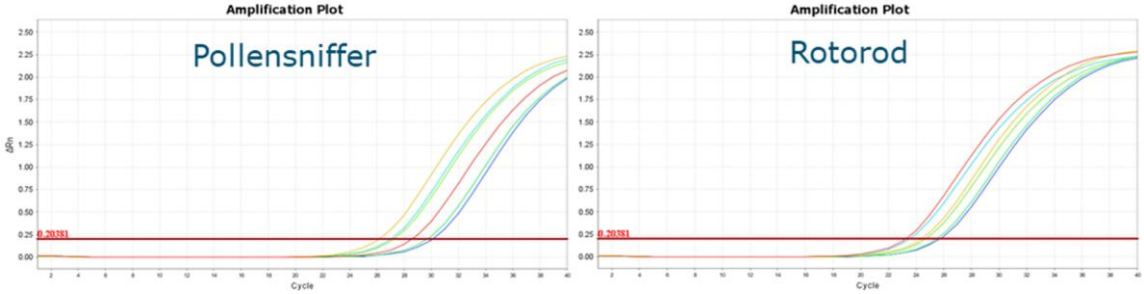


Figure 23. Detection rose powdery mildew (*P. pannosa*) in aerosols collected by a Pollensniffer and Rotorod in a rose greenhouse using qPCR targeting ITS.

Results on detection of both fungal pathogens in air samples collected in the six different greenhouses are shown in Figure 24. The results are expressed as target DNA copies per µl. In total 35 µl of DNA extract was obtained representing an air volume of 14.4 m^3 and 54 m^3 after sampling for 24 hours by the Pollensniffer and Rotorod sampler, respectively. Some samples were collected after 48 h and one sample from the Pollensniffer did not produce qPCR results due to loss of DNA during extraction or sampling failure. To calculate the number of spores the data for powdery mildew (*P. pannosa* and *P. xanthii*) needs to be divided into 100 as one spore contains approximately 100 ITS copies. For detection of *B. cinerea*, the single copy gene β-tubulin was targeted using qPCR. The greenhouses from the two cucumber growers contained the fewest number of spores from both *B. cinerea* and *P. xanthii*, up to 120 and 2 spores per m^3, respectively. The highest levels of *B. cinerea* spores were detected in the gerbera greenhouses, up to 4000 spores per m^3, while very high levels of mildew spores (*P. pannosa*), between 600 and 1000 spores per m^3, were measured in both rose greenhouses and one gerbera greenhouse.

In addition to qPCR assays, the air samples collected in these six greenhouses were also analyzed for the presence of bacterial and fungal communities using metabarcoding targeting 16S and ITS sequences, respectively (see chapter 7 for results).

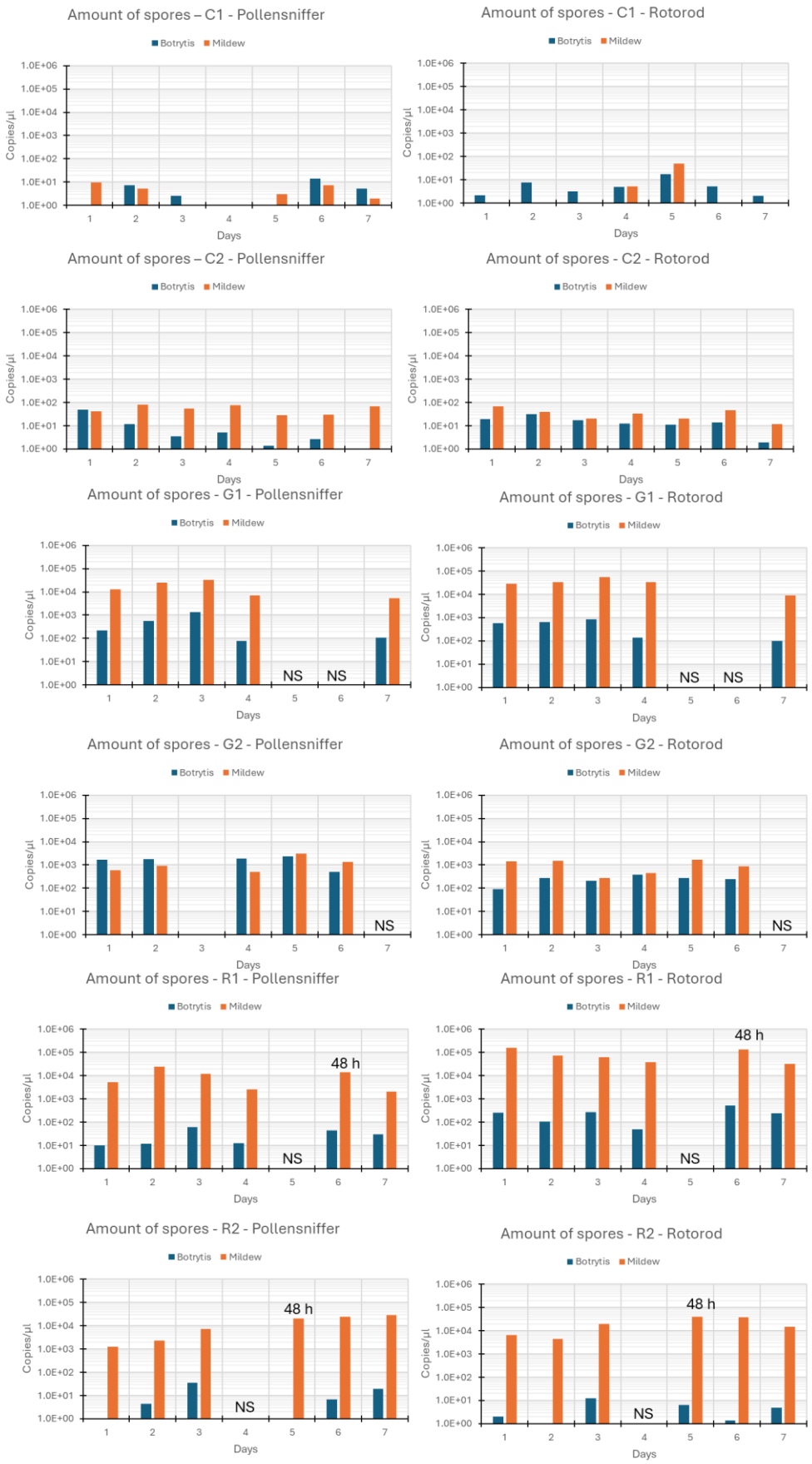


Figure 24. Detection of botrytis and powdery mildew spores in 24 h air samples collected in greenhouses from cucumber, gerbera and rose growers. C1, cucumber grower 1; C2, cucumber grower 2; G1, gerbera grower 1; G2, gerbera grower 2; R1, rose grower 1 and R2, rose grower 2. NS, not sampled. 48 h, 48 h instead of 24 h sampling period.

6.2. Monitoring the long-term dynamics of airborne spores causing powdery mildew and botrytis in greenhouses of rose, gerbera and cucumber using qPCR

After the success of the pilot experiment, long-term monitoring for the presence of airborne spores of the fungal pathogens *P. xanthii*, *P. pannosa*, *B. cinerea* and *M. cucumis* in different greenhouses was carried out using Pollensniffer to collect aerosols in combination with qPCR for quantitative detection. Aerosol samples were collected by growers on different days during the growing season after running the Pollensniffer for 24-h periods. Samples were stored in the freezer until samples were processed for DNA extraction. For all species, pathogen DNA was quantified using qPCR with the primers and probes targeting specific ITS sequences (see Table 3). In comparison with β-tubulin as target, the sensitivity to detect *B. cinerea* in cucumber DNA aerosol samples targeting ITS was approximately 100-fold more sensitive (data not shown). The results for detection of airborne spores of *P. xanthii*, *P. pannosa*, *B. cinerea* in the different greenhouses are shown in Figure 25. Results are expressed as target DNA copies (ITS) per m^3 air. To estimate the number of spores in air samples we assumed that 100 copies of ITS are present in a single spore.



Figure 25. qPCR detection of airborne spores of *P. xanthii*, *P. pannosa*, *B. cinerea* in aerosols sampled in different greenhouses using a Pollensniffer. Results in copies of ITS target DNA per m^3 air. Each day, the Pollensniffer sampled 14.4 m^3 air. One spore contains approximately 100 ITS copies.

Sampling in the rose greenhouse started with a one-year crop. Air samples, 55 in total, were collected by the rose grower during a 253-day period from 20/5/2024 till 28/1/2025. Levels of airborne botrytis spores were continuously high, with between 10^2 and 10^4 spores per m^3 air detected in most aerosol samples. Spores of *P. pannosa* (rose powdery mildew) were also continuously detected, but at lower amounts, mostly between 10 and 10^2 spores per m^3 air. The two samples below the threshold of 100 ITS target copies or one spore per m^3 air are most likely false negatives due to non-functioning of the Pollensniffer or loss of DNA during the extraction steps.

Sampling in the gerbera greenhouse started with a new crop. In total, 56 air samples were collected during a 348-day period, from 16/5/24 till 29/4/25. Levels of Botrytis spores increased gradually from 10 spores per m^3 in May to 10^4 spores per m^3 at the end of November after which it dropped to a level between 10^2 and 10^3 spores per m^3 till the end of the sampling period. Spores of *P. pannosa*, causing mildew on rose, were only detected at very low levels during May till October (data not shown). Levels of spores of *P. xanthii*, known to cause mildew on gerbera, increased gradually from 10 spores per m^3 in May to 10^4 spores in October after which it dropped dramatically to very low levels between 1 and 10 spores per m^3 during December till the end of April.

The presence of airborne mildew and botrytis spores in the cucumber greenhouse was tested in 30 samples, collected during a whole crop cycle from 18/07/24 till 07/11/24 (113 days). Levels of Botrytis were quite constant with most samples containing between 10^2 and 10^3 spores per m^3 of air. Levels of mildew spores were initially very low, around one spore per m^3 air, but increased gradually to over 100 spores per m^3 till the end September after which it dropped to under 10 spores per m^3 till harvest.

Regarding the correlation with airborne spore levels and metadata such as climatical conditions in the greenhouse and activities such as use of crop protection products, harvesting, removing crop debris and cleaning. As the climatic conditions in the greenhouse are kept the same, anthropogenic actions will have the greatest impact on the dynamics of airborne spores in the greenhouse. Application of crop protection products (choice of mode of action, timing and frequency) will have a direct impact on disease levels if no resistance has evolved in the pathogen and needs to be further investigated. Data on application of crop protection products was not available. For the cucumber greenhouse more metadata was available and the lower levels of mildew measured later in the growing season might be due to a decrease in harvesting frequency from 6 to 3 times a week in the second half of the harvesting period (harvest started after 23 days and lasted till the end of the crop cycle).

DNA from a selection of air samples, covering the growing season, was also further analyzed using metagenomics. PCR amplicons to measure the fungal diversity were generated for 25, 29 and 28 air samples collected in the rose, gerbera and cucumber greenhouse, respectively (see chapter 7, section 7.5).

6.3. Detection of *Mycosphaerella cucumis* in cucumber greenhouse aerosols collected by the Pollensniffer

In the last year of the project, we also focused on mycosphaerella in cucumber as this disease is also important for cucumber growers in the Netherlands. *Mycosphaerella cucumis* (aka *Didymella bryoniae* or *Stagonosporopsis cucurbitacearum*) can be seed-borne and is a destructive homothallic fungus that attacks stems, leaves, and fruits from cucumber and other members of the cucurbit family, including melons, squash, and pumpkins. Its fruiting bodies appear as black spots (pycnidia and/or perithecia) on infected lesions, accompanied by a golden-brown gummy exudate, hence the name gummy stem blight, and tissue necrosis. In cucumber, the most important symptom is internal fruit rot, which is hard to detect during harvest and sorting as it only, but not always, can be recognized from the outside by the small narrowing of the tip of the mature fruits (Figure 26).



Figure 26. Internal fruit symptoms and an isolate of *Mycosphaerella cucumis* growing on yeast extract peptone dextrose (YPD) agar

To control disease, sanitation, removal of all plant debris and reducing the humidity are all important, as the fungus overwinters on infected plant residues and free moisture on leaf surfaces is required for spore germination. Use of disease-free seeds, resistant varieties, application of crop protection products and plucking of flowers, preventing entry of the fungus to the fruit after (airborne) spore arrival, are other key measures to prevent disease and to maintain yield and quality.

The air samples collected previously by the Pollensniffer in the cucumber greenhouse during a whole crop cycle, lasting from 18/07/24 till 07/11/24 (113 days), were also tested for presence of *M. cucumis* (*D. bryoniae*) using qPCR assays developed by Ling *et al*. (2010) (Figure 27).

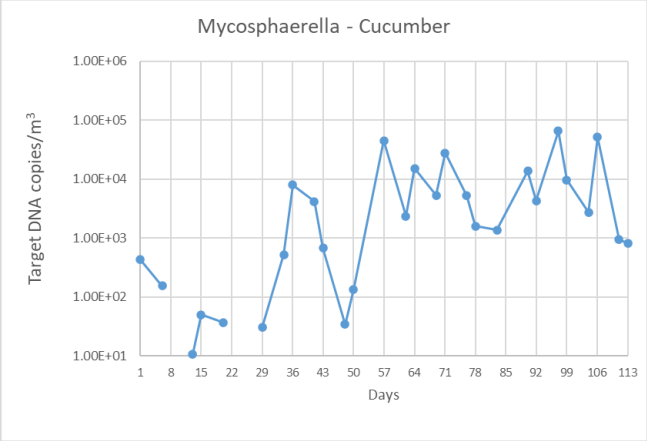


Figure 27. qPCR detection of airborne spores of *Mycosphaerella cucumis* (*Didymella bryoniae*) in aerosols sampled in a cucumber greenhouse using a Pollensniffer. Results in copies target DNA (sequence of RAPD-fragment) per m^3 air. The Pollensniffer sampled 14.4 m^3 air each day.

Although it is not known how many copies of the RAPD fragment are present in one spore, we can see that the number of airborne spores increases after approximately one month and further over time during the growing season. This trend is different from *B. cinerea* where a constant spore concentration was measured during the growing season or from *P. xanthii* where we saw first an increase and later a decline in spore levels.

The primers and probe from the qPCR assay detect two distinct genetic groups of *D. bryoniae* isolates, RGI and RGII, based on RAPD sequences (Ling *et al*., 2010). Although *D. bryoniae* originally corresponded to *S. cucurbitacearum*, more recent studies have revealed that gummy stem blight on cucurbits is caused by three different *Stagonosporopsis* species: *S. cucurbitacearum*, *S. citrulli* and *S. caricae.* The latter species is believed to represent the ancestral lineage and the only species that can infect papaya and babaco-mirim. The sister species *S. citrulli* and *S. curbitacearum* correspond to *D. bryoniae* RGI and RGII, respectively (Stewart *et al*., 2015). Using RGI and RGII specific primers described by Somai *et al*. (2002), a selection of cucumber greenhouse aerosol samples was tested with end-point PCR to see which fungal species is present. As shown in Figure 28, most samples tested positive for the presence of *S. citrulli*, while *S. cucurbitacearum* was absent.

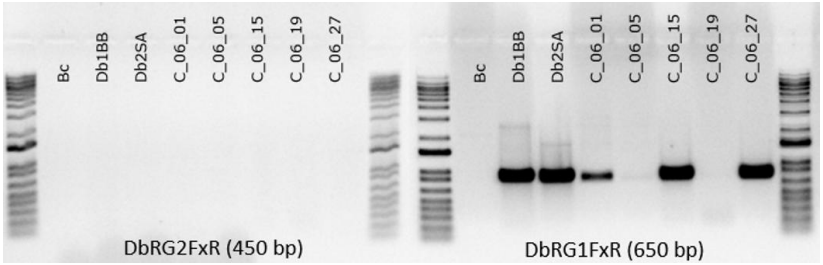


Figure 28. PCR amplification of RAPD-fragments specific to *S. cucurbitacearum* (RGII, left) and *S. citrulli* (RGI, right). DNA samples: Bc (*Botrytis cinerea*), Db 1BB and Db 2SA are *D. bryoniae* strains isolated from cucumber fruits, C-06-01, 05, 15, 19 and 27 are air samples from the cucumber greenhouse. C-06-05 contains a very low level of *D. bryoniae* while sample C-06-19 contains PCR inhibiting compounds (brown DNA pellet visible upon extraction).

6.4. Detection of *Botrytis cinerea*, *Podosphaera xanthii* and *Stagonosporopsis citrulli* in cucumber greenhouse aerosols collected by the Coriolis Compact air sampler

In the last year of the project, the use of the Coriolis Compact was also evaluated for air sampling and detection of fungal pathogens in greenhouses where cucumbers are grown. Coriolis Compact is a portable air sampler, suitable to operate in both indoor and outdoor environments. It can run autonomously for 8 h on a built-in battery and works as a dry cyclone (Figure 29). Its innovative dry cyclonic technology aspirates the particles with an airflow of 50 liters per minute and centrifuges them in a cone. The collected aerosols containing microorganisms (e.g. viruses,

bacteria and fungi), mainly in the size range from 500 nm to 10 µm, can then be recovered by rinsing the cone with an appropriate buffer so that DNA can then be extracted and sample further analyzed with molecular methods.



Figure 29. The Coriolis Compact device sampling air in a cucumber greenhouse

The Coriolis Compact was first used together with a Rotorod sampler in greenhouse with cucumbers where air samples were collected daily for 24 or for 72 hours by sampling over by the weekend. Assuming one spore contains approximately 100 ITS copies, the highest levels of airborne spores were detected for *B. cinerea* (up to 120 spores per m^3 air using the Coriolis Compact)) while *P. xanthii* was only detected at very low levels (up to 2 spores per m^3 air using the Coriolis Compact) using qPCR (Figure 30). *Mycosphaerella cucumis* (*D. bryoniae*) was not detected in any of the samples as qPCR assays were all negative after 40 amplification cycles. In comparison with Rotorod, higher levels of spores were generally detected for both pathogens using the Coriolis Compact.

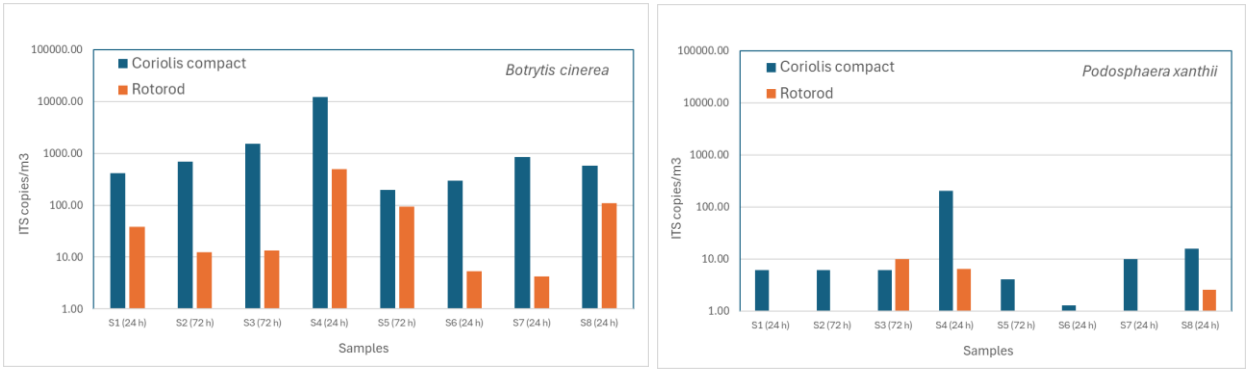


Figure 30. Detection of airborne spores of *B. cinerea* and *P. xanthii* in aerosols sampled in an experimental cucumber greenhouse using a Coriolis Compact and Rotorod sampler during 7/11/25 till 20/11/25. After correction for sampling duration, results expressed as number of ITS target DNA copies per m^3 air.

The second application of the Coriolis Compact was in two commercial cucumber greenhouses (A and B) with different levels of mycosphaerella-infected plants. In total, 1 and 190 infected plants were observed in greenhouse A and B, respectively, in week 20 of 2026. The Coriolis compact was deployed for different durations on eight different occasions (four times in each greenhouse at different positions) during weeks 19 and 20 (Table 6). *Mycosphaerella cucumis* was detected on all air samples from greenhouse B where high numbers of infected plants were observed in week 20. In contrast with greenhouse B, all four air samples from greenhouse A tested negatively for the presence of *M. cucumis*. Assuming the targeted RAPD fragment is present as a single copy in the *M. cucumis* genome, the DNA copy number m^{-3} can be directly translated into spore numbers, ranging from 0.79-20.69 spores per m^3 of air.

Isolation and further characterization of three *M. cucumis* strains, two obtained from different infected fruits of greenhouse B as well as one strain from an infected leaf of greenhouse A, showed that all three isolates can be designated as *S. citrulli* after ITS sequencing and DbRG1FxR PCR amplicon analysis.

Table 6. Air sampling and detection of *Mycosphaerella cucumis* in two commercial cucumber greenhouses in 2026. Ct is detection cycle in qPCR; nd, no detection after 40 PCR amplification cycles. A specific RAPD fragment of *M. cucumis* (*D. bryoniae*) is targeted by qPCR (see Table 4).

Week	Date	Time	Hours	Air (m^3)	Greenhouse	Sample	Position	PCR (Ct)	DNA copies m^{-3}
19	8-5 till 9-5	13:30 - 05:00	15.5	46.5	A	S1	1	nd	0.00
19	9-5 till 10-5	05:00 - 14:30	35.5	106.5	A	S2	1	nd	0.00
19	10-5 till 11-5	14:30 - 07:30	17	51	A	S3	1	nd	0.00
20	11-5 till 11-5	07:30 - 15:00	7.5	22.5	A	S4	1	nd	0.00
20	12-5 till 13-5	07:00 - 07:00	24	72	B	S5	2	34.45	6.27
20	13-5 till 13-5	07:30 - 15:00	7.5	22.5	B	S6	2	34.41	20.69
20	13-5 till 14-5	15:00 - 15:00	24	72	B	S7	3	37.21	0.79
20	14-5 till 15-5	15:00 - 07:30	16.5	49.5	B	S8	4	35.88	3.13

6.5. Detection of *Botrytis cinerea* in greenhouse aerosols using ultra-fast on-chip qPCR assays

Ultra-fast on-chip qPCR assays were also carried out with DNA samples from greenhouse aerosols sampled with the Pollensniffer (see Figure 25). The main purpose was to see if the assays worked without inhibition being observed. Three different samples from both the gerbera and the cucumber greenhouse, containing low intermediate and high levels of pathogen DNA, were selected that previously were tested using qPCR and had shown different amounts of pathogen target DNA (Table 7).

Table 7. Selection of DNA samples representing greenhouse aerosols sampled with the Pollensniffer and previously tested for presence of *Botrytis cinerea* using qPCR targeting ITS. Ct is detection cycle of qPCR. Results expressed in copies of ITS target DNA obtained by running g-block standard curve samples. One spore contains approximately 100 ITS copies.

Greenhouse samples	Sampling date	qPCR (Ct)	ITS DNA copies	ITS DNA copies m^{-3} air
Gerbera				
G04-009	03/07/24	31.0	83.7	$2.03*10^2$
G04-011	10/07/24	26.0	2460	$5.98*10^3$
G04-030	14/11/24	21.5	73646	$1.79*10^5$
Cucumber				
C06-003	25/07/24	29.3	270.8	$6.58*10^2$
C06-006	06/08/24	23.5	12348	$3.0*10^4$
C06-019	26/09/24	20.6	146092	$3.55*10^5$

The results of the ultra-fast on-chip qPCR assays, performed in triplicate are shown in Figure 31.

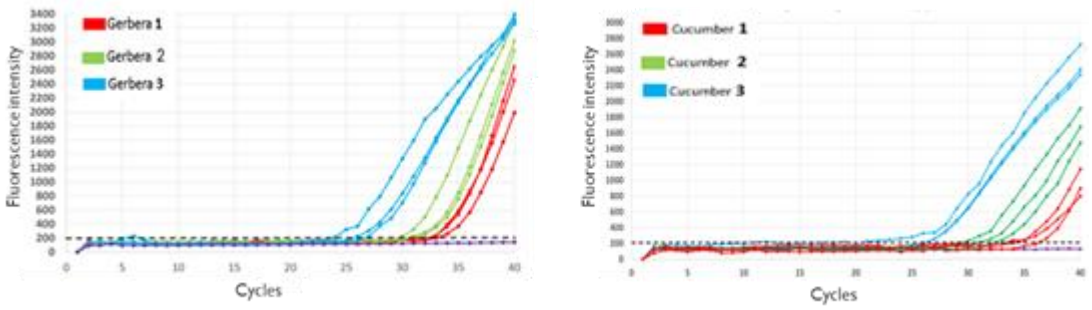


Figure 31. Detection of *Botrytis cinerea* in DNA samples from greenhouse aerosols within 10 minutes using Ultra-fast on-chip qPCR assays. Gerbera aerosol DNA samples 1, 2 and 3 were derived from samples G04-009, G04-011 and G04-030 and cucumber 1, 2 and 3 from C06-003, C06-006 and C06-019 (see Table 7).

The results of the on-chip qPCR assays using the Takara Speed STAR HS PCR kit agreed with those from the qPCR assays using the Quantabio PerfeCTa ToughMix as the amount of target DNA increased for the greenhouse aerosol DNA when samples 1 to 3 were tested. Based on the shape of the PCR amplification curves, there was no sign that inhibition was an issue during DNA amplification.

7. Metabarcode-based detection of fungal pathogens in aerosols sampled from commercial gerbera, rose and cucumber greenhouses

7.1. Airborne eDNA analysis

Detection and identification of airborne microorganisms was previously dependent on the use of culture-based techniques and microscopy. The development of molecular tools such as PCR and next-generation sequencing (NGS) now offers the opportunity to collect vast amounts of data on airborne environmental DNA (eDNA). An eDNA analysis workflow has four different steps that each needs to be optimized depending on the research objectives. It starts with sample collection, followed by DNA extraction and isolation, DNA amplification and sequencing and ending with using a bioinformatics pipeline. Figure 32 presents an overview as published by Berelson *et al*. (2025).

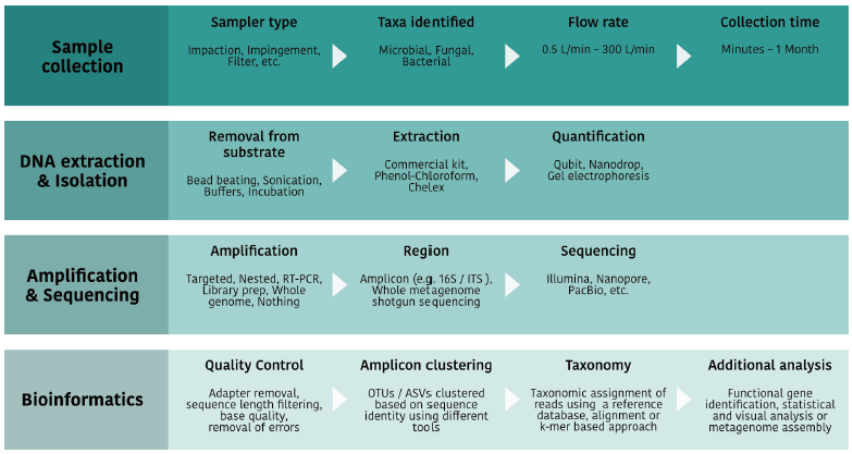


Figure 32. Overview of eDNA analysis workflow based on four different stages (Berelson *et al*., 2025)

For this project, primers for PCR amplification of fungi and bacteria have been selected for both the Oxford Nanopore Technologies (ONT) sequencing and the Illumina sequencing platforms for long and short reads, respectively (Figure 33). For detection of bacteria 16S rRNA with the nine hypervariable regions was targeted while for fungi the variable Internal Transcribed Spacer regions ITS1 (located between the 18S ribosomal subunit (SSU) and 5.8S rRNA genes) and ITS2 (located between the 5.8S rRNA and 28S genes), were amplified and sequenced.

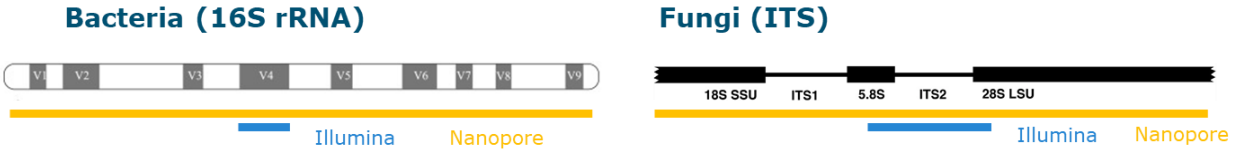


Figure 33. Overview of DNA target sequences for detection and identification of bacteria (left) and fungi (right).

7.1. Evaluation of high-throughput Illumina sequencing as a monitoring technique for plant pathogens in greenhouses

For Illumina sequencing the bacterial 16S rRNA V4 region of 291 bp, the underlined primers with MiSeq adaptors MSAf-B-515f (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3') and MSAr-B-806r (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3') were used (Wasimuddin *et al*., 2020). Primers MSAf-gITS7 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCGARTCTTTG-3') and MSAr-ITS4 (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3'), were used to amplify a 292 bp fragment covering the fungal ITS2 region (Viotti *et al*., 2024). To evaluate the Illumina sequencing tools, we generated mock bacterial and fungal communities, including several target pathogens, from isolate DNA stock solutions in equal and unequal concentrations, and analyzed the resulting test samples with Illumina. The results of Illumina sequencing of mock communities are shown in Figure 34.

Aerosol DNA samples from the greenhouse pilot inoculation experiment with pathogen mixtures (see chapter 5.3) were also tested with Illumina sequencing and compared with qPCR (Table 8).

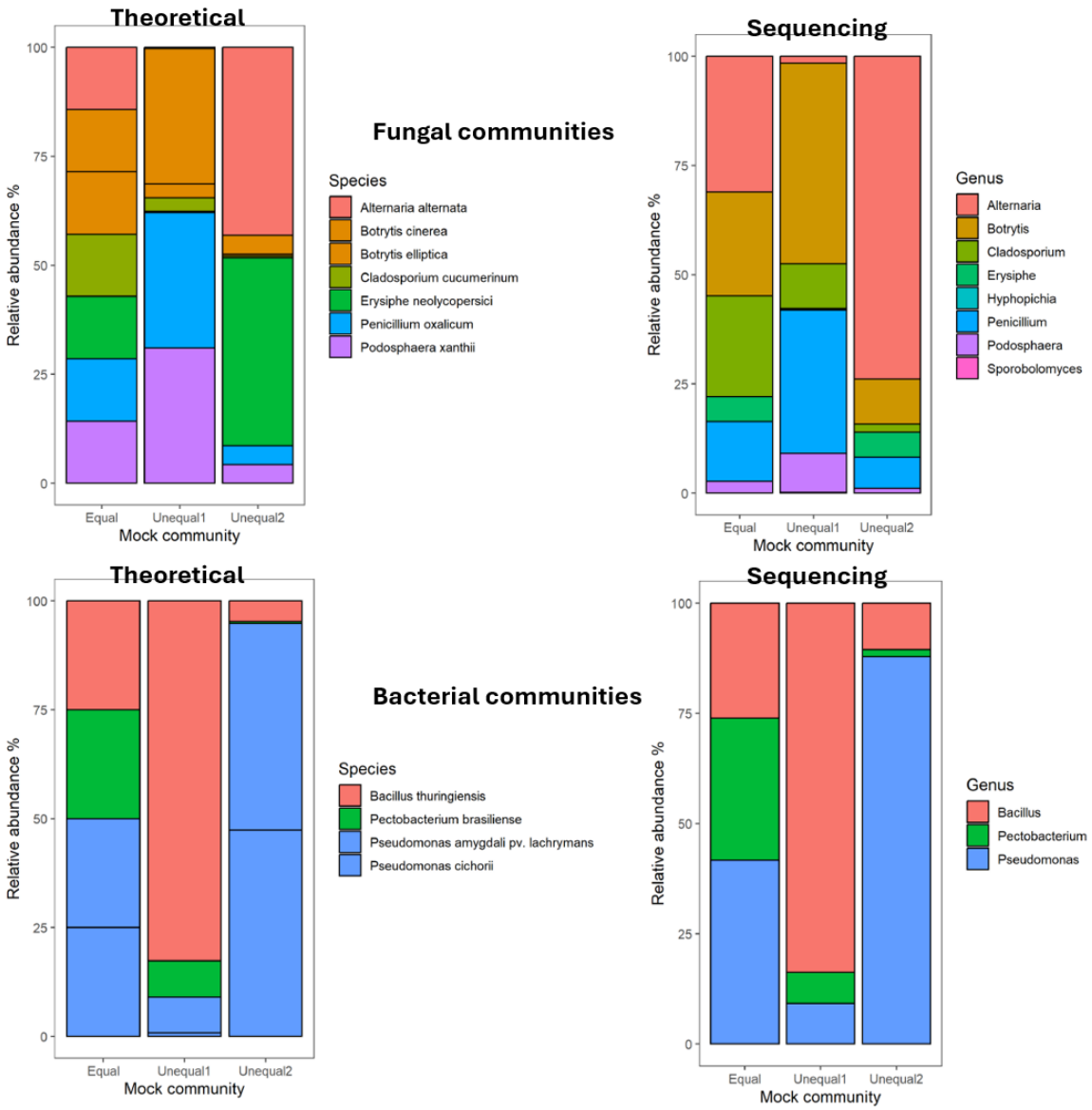


Figure 34 Relative abundance of fungi (top) and bacteria (below) of mock communities measured using Illumina sequencing; Theoretical: expected composition in one mix of equal and two mixes of unequal proportions; Sequencing results: relative abundance recovered by sequencing.

Table 8. Relative abundance of *Podosphaera xanthii*, *Botrytis cinerea* and *Pseudomonas amygdali* (*syringae*) pv. l*achrymans* in aerosols sampled with different air sampling devices using Illumina sequencing and qPCR. qPCR targeting β-tubulin (*P. xanthii* and *B. cinerea*) and HrpZ (*P. amygdali* pv. *lachrymans*)

Air sampler	Species	Relative abundance (%)	qPCR (Ct)	DNA copies µl^{-1}
Rotorod	*Podosphaera xanthii*	37.20	33.73	4.33
Pollensniffer	*Podosphaera xanthii*	41.00	33.51	7.99
Coriolis Micro	*Podosphaera xanthii*	1.10	Not detected	Not detected
Rotorod	*Botrytis cinerea*	32.40	32.21	127.95
Pollensniffer	*Botrytis cinerea*	37.20	33.40	124.95
Coriolis Micro	*Botrytis cinerea*	2.70	36.54	5.06
Rotorod	*P. amygdali* pv. *lachrymans*	24.00	27.90	3710.08
Pollensniffer	*P. amygdali* pv. *lachrymans*	8.20	31.05	316.33
Coriolis Micro	*P. amygdali* pv. *lachrymans*	39.10	27.04	5165.52

Results of Illumina sequencing of mock communities showed that all target fungi and bacteria could be recovered (Figure 34). The representation of relative abundances was quite accurate for most species on genus level.

However, sequencing short sequences and the use of reference databases did not allow to distinguish fungi and bacteria at species level.

Illumina sequencing of DNA samples from aerosols sampled in the greenhouse pilot inoculation experiment with pathogen mixtures correlated well with qPCR (Table 8). As observed before in chapter 5.3, the Coriolis Micro was efficient in capturing small particles, represented by *P. amygdali* pv. *lachrymans*, while larger fungal spores were not (*P. xanthii*) or only in very small quantities detected (*B. cinerea*). The Pollensniffer and Rotorod were both efficient in capturing fungal spores, with the Rotorod in comparison with the Pollensniffer more efficient in capturing the smaller particles of *P. amygdali* pv. *lachrymans*.

7.2. Development of Nanopore sequencing-based metabarcoding protocols for detection of bacteria and fungi in aerosol DNA samples

Because more microorganisms can be identified using longer DNA sequences, Nanopore sequencing protocols were further developed for their application in MinION or GridION devices. Nanopore-sequencing is a third-generation sequencing technology that detects changes in electrical current, as DNA strands pass through a protein nanopore, embedded in a membrane. During the project, both the methodology, involving DNA extraction and optimal PCR amplification of DNA sequences specific to bacteria (16S ribosomal RNA gene (16S-rRNA) and fungi (the Internal Transcribed Spacer (ITS) regions located between the 18S and 28S rRNA genes in the rDNA cluster) and the bioinformatics pipeline (an automated series of steps required to collect large amounts of data, DNA sequences, to be processed and analysed) were continuously further improved.

The preparation for Nanopore sequencing involves in short the following steps: barcode attachment, where amplicons are given a sequence tail unique for each sample. Protein adapters are ligated to the ends of the barcode fragments for guidance to a nanopore. The prepared DNA library is loaded onto a specialized flow cell with around 1500 pores and with a reading speed of 4000 bases a second. A voltage is applied across the membrane, and as each DNA molecule passes through a nanopore, it causes a change in electrical conductance that is detected by the sequencer. This signal is used to identify the nucleotide sequence of DNA molecules. This process is called base-calling, and it produces a series of fragments representing the sequence of the DNA amplicons. The analyzing of the data is done by using Galaxy and R-studio as Graphical User Interface (GUI). With Galaxy, different tools can be used on the dataset. There are tools such as Nanoplot for sequence quality control. After this the primers can be trimmed using the DADA2 tool. UNOISE can be used for clustering and making OTU's (Operational Taxonomic Units) or ASV's (Amplicon sequence Variants) (see Figure 35 for flowchart).

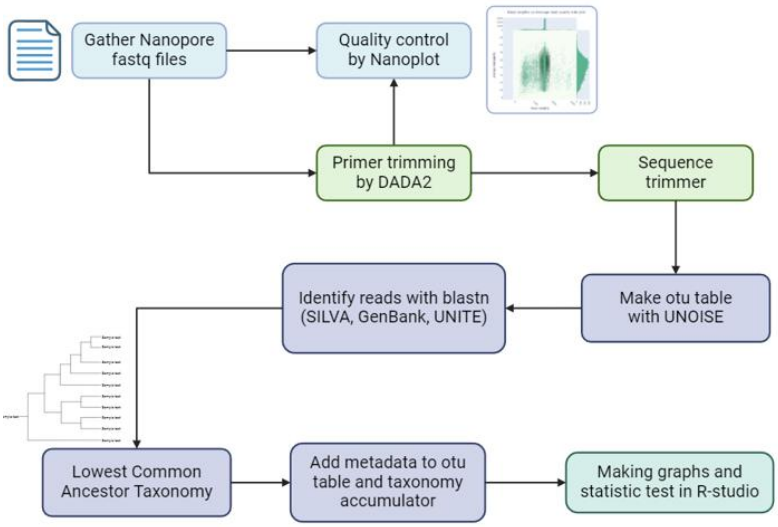


Figure 35. A flowchart with all the tools used on the generated data with the UNOISE method. GUI used are Galaxy and R-studio. Flowchart was created in Biorender.

The OTU's or ASV's can also be identified using BLAST against reference databases like GenBank, UNITE, RefSeq and SILVA for the taxonomy identification. Kraken2 can be used without OTU or ASV step for direct identification odd all sequences against the RefSeq database. After this a lowest common ancestor (LCA) tool was used for bacteria and fungi that can't been identified completely to species level. Lastly a tool was used to combine the LCA-file and OTU-table. After the analysis in Galaxy, R-studio can be used for making graphical images.

7.3. Application of Nanopore sequencing to identify airborne bacteria and fungi in aerosols sampled from the greenhouse inoculation experiment and on a drone

For detection and identification of bacteria and fungi in aerosols using Nanopore sequencing, we first focused on the air samples that were collected in the greenhouse pilot inoculation experiment (section 5.3), on a drone in and outside an anthurium greenhouse (section 5.6) and in commercial greenhouses of cucumbers, gerberas and roses (section 6.1) using different air sampling devices. PCR reactions were carried out using the Thermo Scientific™ Phire Hot Start II DNA polymerase enzyme (see Appendix 3) using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'), amplifying a fragment of 1400-1500 bp that covers almost the full-length 16S rRNA sequence (Heuer *et al.*, 1997). For detection of fungi, primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), amplifying fragments of 300-500 bp covering the fungal ITS2 region (White *et al.*, 1990), were used (see Figure 36).

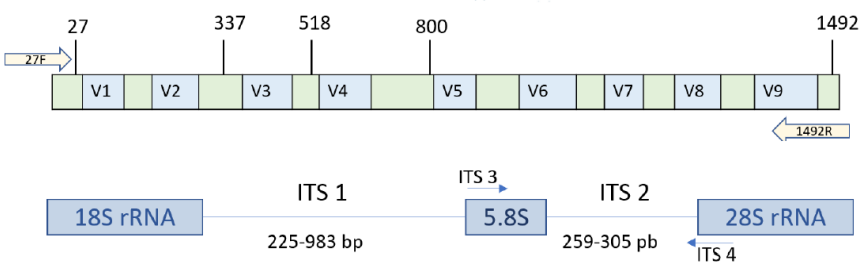


Figure 36. Overview of PCR targets for bacteria (16S rRNA) and fungi (ITS2) and corresponding primer positions.

PCR amplicons were analyzed on gel using the Qiaxcel system. The purification was done by using the NucleoMag® NGS Clean-up and Size Select kit. The measurement of the DNA concentration was done by using the Qubit, with the dsDNA BR Assay. The cleaned up 16S amplicons were first diluted to 200 fmol. The library prep was made by using the Native Barcoding Kit 96 V14 (SQK-NBD114.96) and the sequencing run was done using the GridION in combination with a flowcell type R10.4.1.

Figure 37 shows the results of the 16S-rRNA gene Nanopore sequence analysis in aerosols sampled in the experimental greenhouse and on a drone in and outside an anthurium greenhouse.

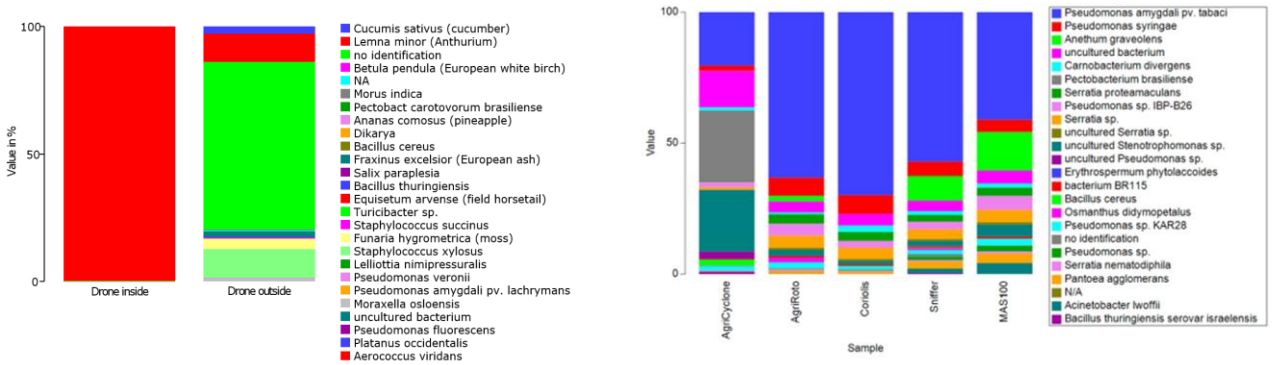


Figure 37. Relative abundance bar charts of 16S-rRNA gene analysis of air samples collected in and outside an anthurium greenhouse using a drone with Rotorods (left) and in an experimental greenhouse using different sampling devices (right).

From the results it is clear that 16S rRNA gene-targeting primers can cross-react with eukaryotic DNAs, including chloroplast DNA from plant pollen. Chloroplast DNA is most likely originally derived from an ancestral endosymbiotic cyanobacterium. Most plant derived sequences came from Anthurium (*Lemna minor* is a close relative and has identical reads) and cucumber (*Cucumis sativus*), likely due to proximity of cucumber greenhouses nearby. Detection of European ash (*Fraxinus excelsior*), European white birch (*Betula pendula*) and mulberry (*Morus indica*) likely occurred through capture of pollen, while *Funaria hygrometrica*, a moss that was detected, produce spores. Bacteria, including *Pseudomonas*, *Bacillus*, *Pectobacterium*, *Turicibacter*, *Staphylococcus* and *Aerococcus* species were also identified. Detection of the gram-negative rod-shaped *Acinetobacter Iwolffii* and gram-positive spherical *Staphylococcus xylosus* can be explained by contamination of Rotorods by human DNA through handling as these bacteria are commonly found as a member of the skin flora. Many sequences could not be identified.

The abundance bar chart confirmed the results from the targeted detection, with efficient capture of *P. amygdali pv. lachrymans* (same sequence as *P. amygdali* pv. *tabaci*) particles (typical size 1-2 µm), released as part of the mixed inoculum experiment in the greenhouse, using the Coriolis, Rotorod, Pollensniffer and MAS-100 air sampling devices. The cyclone was least efficient in sampling *P. amygdali* pv. *lachrymans*.

The results of the fungal ITS2 DNA identification in aerosol DNA samples from in the experimental greenhouse are shown in Figure 38.

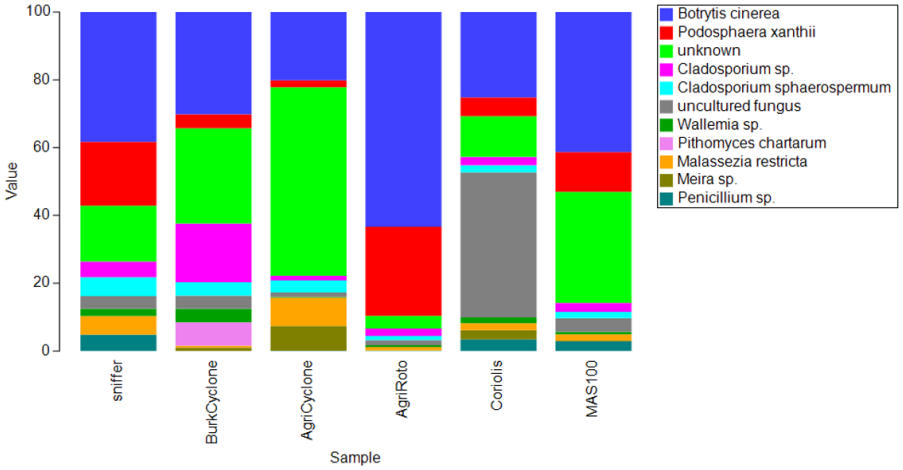


Figure 38. Fungal ITS2 sequence identification results (%) of air samples collected in an experimental greenhouse by different sampling devices using Unoise Blast Genbank.

As expected from the targeted detection using qPCR, most fungal ITS2 DNA reads originated from the inoculated spores from *B. cinerea* and *P. xanthii*. Most of this inoculum was captured by the Rotorod, followed by the Pollensniffer and MAS-100 air sampling devices. Low amounts of these target sequences were found in aerosols sampled by the Coriolis and the two different cyclone devices. Most devices have also sampled spores belonging to *Penicillium* and *Cladosporium* species, airborne fungi commonly found in air samples. The presence of *Malassezia restricta*, a basidiomyceteous yeast and one of the most prevalent species on human skins, is probably due to contamination when handling the devices and/or during sample processing.

7.4. Application of Nanopore sequencing to detect and identify airborne bacteria and fungi in aerosols sampled from the pilot experiment with commercial greenhouses

16S rRNA and ITS2 Nanopore sequencing was also carried out with the aerosol DNA samples that were collected from different rose, cucumber and gerbera greenhouses during a one-week pilot experiment (see section 6.1). Visible PCR 16S rRNA and ITS2 amplicons could not be generated from all samples (Table 9).

Table 9. Visible PCR amplification of 16S rRNA and ITS2 DNA target sequences from aerosols sampled in different commercial greenhouses with roses, gerberas and cucumbers

		Rose 1	Rose 2	Gerbera 1	Gerbera 2	Cucumber 1	Cucumber 2
16S	Pollensniffer	5/6	5/6	0/6	1/6	1/7	3/7
	Rotorrod	4/6	5/6	2/6	1/6	1/7	3/7
ITS	Pollensniffer	5/6	5/6	2/6	1/6	1/7	5/7
	Rotorrod	5/6	5/6	4/6	1/6	6/7	7/7

The amount of target DNA is likely to affect the species' richness. With only low amount of target DNA, the proportion of amplicons are due to contamination by DNA from airborne microbes and other particles. Contamination by human and eDNA through sample handling is also possible. Figure 39 shows the species richness of fungal and bacterial communities in the DNA samples based on reads from ITS2 and 16S rRNA amplicons, respectively.

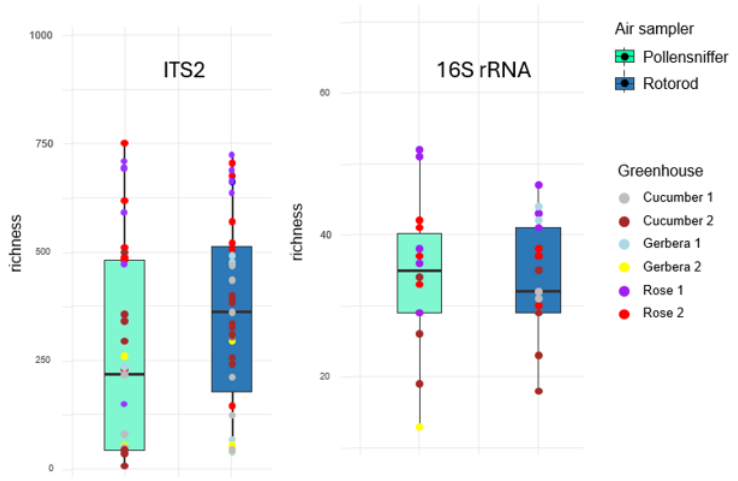


Figure 39. Comparison of species richness based on fungal ITS2 short reads (left) and bacterial 16S rRNA long reads (right) in air samples collected by Rotorod and Pollensniffer samplers using Nanopore sequencing. The dataset is made with Galaxy using the UNOISE algorithm and visualized with R-studio. Each dot is a sample; the color of the dot indicates a greenhouse, and the color of the boxplot shows the air sampler.

Upon comparing the two air samplers, no clear differences were observed between the Pollensniffer and the Rotorod. The air samples collected in the rose greenhouses with the highest proportion of visible amplicons showed also the highest species richness. As evident from the figure, there is considerable overlap between the box plots and samples, with fungal richness detected at significantly higher levels than bacterial richness.

Figure 40 shows in more detail the most abundant 16S rRNA reads from the different greenhouses.

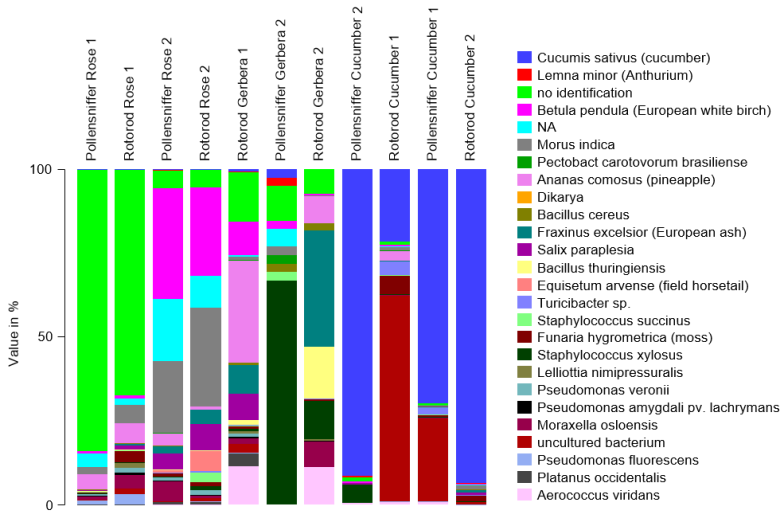


Figure 40. Results of the 16S rRNA-gene amplicons visualized with primer-E. This relative abundance bar plot shows for each greenhouse and air sampler the presence of different bacterial species. The dataset is made with Galaxy with the use of UNOISE.

A large proportion of 16S rRNA could not be identified, especially in the samples from the rose greenhouses. As expected, due to the bacterial evolutionary origin of chloroplast DNA, host plants, as well as other plants, especially trees (e.g., birch, ash and willow), were also detected due to pollen release. The Rotorod and Pollensniffer samples showed similar results for most greenhouse samples, with exception of the gerbera greenhouse samples. No reads were obtained for the gerbera 1 greenhouse with the pollensniffer, while results for gerbera 2 were completely different. High amounts of reads from *Staphylococcus xylosus*, a gram-positive bacterium commonly found in skin flora, in the Pollensniffer samples collected in the gerbera 2 greenhouse is likely due to contamination due to human handling during sampling and processing as low amounts of template DNA were present in these samples.

The results for the ITS2 reads are shown in Figure 41. The list of fungal species is not complete, the complete list of fungal species that were identified using ITS2 as marker is shown in Appendix 4.

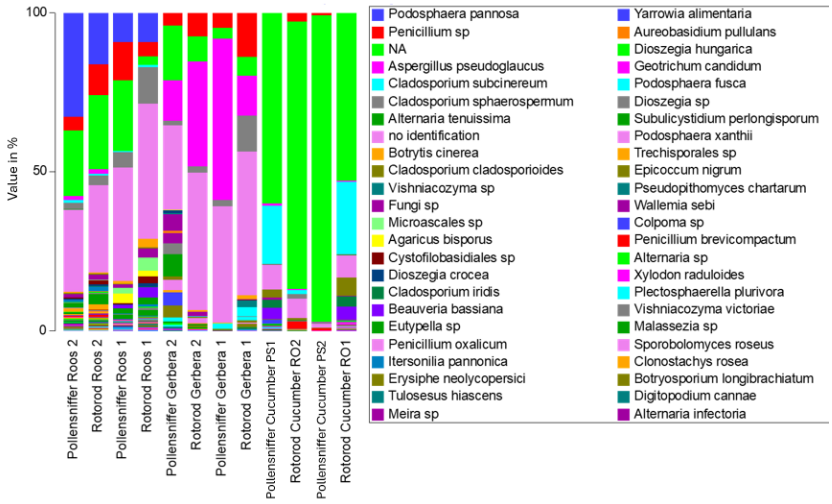


Figure 41. Results of the ITS2 region amplicons visualized with primer-E. This relative abundance bar plot shows for each greenhouse and air sampler the presence of fungi. The dataset is made with Galaxy with the use of UNOISE.

For both rose greenhouses, high number of ITS2 reads were recorded in both Rotorod and Pollensniffer air samples for rose itself (mainly *Rosa chinensis*) and for saprophytic fungi like *Aspergillus pseudoglaucus*, *Cladosporium sphaerospernum* and *Penicillium steckii* and other members belonging to these genera. As expected, a high number of reads were found for the key plant pathogens *P. pannosa* and *B. cinerea*, but *Didymella pinodella* was also found often. Furthermore, common mushrooms (*Trametes versicolor* and *Agaricus vaporarius*), yeasts (*Sporobolomyces roseus* and *Dioszegia hungarica*), *Beauveria bassiana* (entomopathogenic fungus) and *Clonostachys rosea* (mycoparasitic fungi) were also frequently found. The latter species can be used as crop protection agents in the greenhouse, but both *Clonostachys* spp. and *Beauveria* spp. can also naturally occur in many environments.

Not many Pollensniffer and Rotorod air samples from the gerbera greenhouses yielded enough reads. Gerbera was detected as well as species of *Aspergillus* (*A. westerdijkiae*) and *Penicillium* (*P. fluviserpens*) and the key plant pathogens *P. xanthii* (*P. fusca*) and *B. cinerea*, together with *Plectosphaerella* sp..

For the cucumber greenhouses, large numbers of reads were found for *Cladosporium* spp., including *C. iridis* and *C. sphaerospernum*. *Cladosporium* spp., including *C. sphaerospernum* are commonly found in cucumber bioaerosols (Xiong *et al*., 2025). *Alternaria* (*A. infectoria* and *A. alternata*), *Saccotheciaceae* spp., *Erysiphe alphatoides* (causal agent of oak powdery mildew) and the key cucumber pathogens *P. xanthii*, *B. cinerea* and *Stagonosporopsis cucurbitacearum* (*M. cucumis*) were also found, albeit at much lower read numbers. *Beauveria bassiana* was also found in large numbers on several days, but only in greenhouse cucumber 1. Large numbers of reads for *Penicillium brevicompactum* were only found in cucumber 2 greenhouse. The latter fungus is not a cucumber pathogen, but a common mold fungus that can be found in cucumber greenhouses as part of the normal bioaerosol environment (Hansen *et al*., 2012).

7.5. Development of long-read ITS1+2 Nanopore sequencing protocol to identify fungi in aerosols

During the last phase of the project, a protocol was developed to obtain long-read sequencing targeting ITS1 and ITS2 simultaneously to obtain a better resolution to distinguish different and more closely related fungal species. In total 4 different primer sets with 2 different forward and reverse primers with similar annealing temperatures were tested and compared (Figure 42).

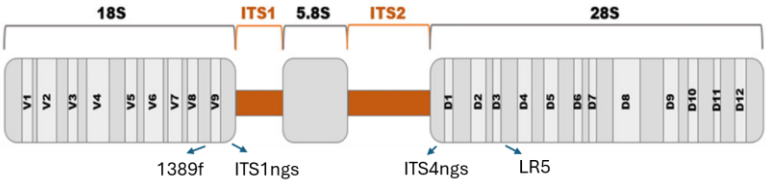


Figure 42. Overview of the 18S (forward) and 28S (reverse) primer positions to create ITS1+2 amplicons (figure modified from D'Andreano *et al*. (2020)).

The sequences of the selected primers are: 5'-TTGTACACACCGCCC-3' (1389f), 5'-TCCGTAGGTGAACCTGC-3' (ITS1ngs), 5'- TCCTSCGCTTATTGATATGC-3' (ITS4ngs) and 5'-TCCTGAGGGAAACTTCG-3 (LR5). After testing DNA from six soil samples, one air sample and a positive control with *Fusarium oxysporum*, with the four primers 1389f/ITS4ngs (~800 bp), 1389f/LR5 (~1600 bp), ITS1ngs/ITS4ngs (~650 bp) and ITS1ngs/LR5 (~1400 bp), primer set 1389f/ITS4ngs was selected due to its consistent and higher yield of amplicons around its expected size in the range of 600-1000 bp in comparison with the other primer sets (Figure 43).

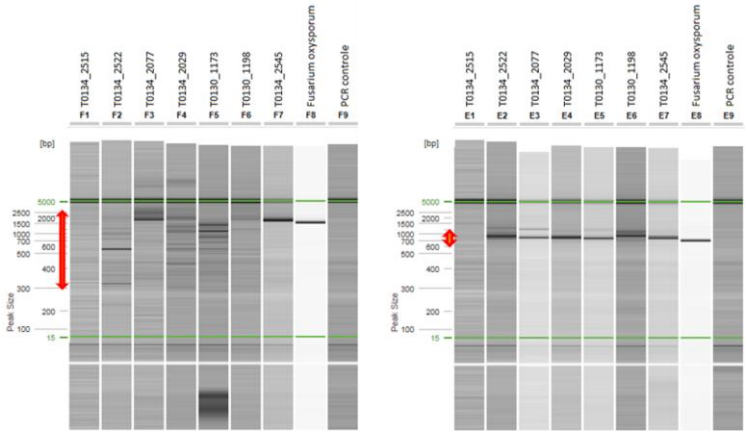


Figure 43. Analysis of PCR amplicons derived from primer sets 1389f/LR5 (left) and 1389f/ITS4ngs (right) using the Qiaxcel system. DNA samples from soil (lanes 1-6), air (lane 7) and controls (lanes 8-9)

Further analysis of the air sample using OTU Blast ID searches showed that primer set 1389f/ITS4ngs was better to distinguish fungal species than short-read ITS2 and ITS1+2 PCR amplicon sequence analysis using primer sets ITS3/ITS4 and ITS1ngs/ITS4ngs, respectively (data not shown). *Fusarium oxysporum* species identification was also improved using Kraken2 ID as ITS1+2 provides a larger, more informative barcode region than ITS2, overcoming the high sequence similarity shared across Fusarium species complexes.

7.6. Application of long-read ITS1+2 Nanopore sequencing to investigate the long-term dynamics of fungal communities in aerosols from cucumber, gerbera and rose greenhouses

To follow the long-term dynamics of fungal communities in aerosols from cucumber, gerbera and rose greenhouses, a selection of DNA samples covering the life span of the different greenhouse aerosol samples described section 6.2, being cucumber (113 days), gerbera (348 days) and rose (253), were further tested. Unfortunately, although the qPCR measurements didn't give problems with PCR amplification of small target amplicons (~100 bp) of selected fungal pathogens using PerfeCTa ToughMix, inhibition was observed while amplifying the larger 1389f/ITS4ngs amplicons (~800 bp) from air samples using different PCR DNA polymerase mixes (Figure 44).

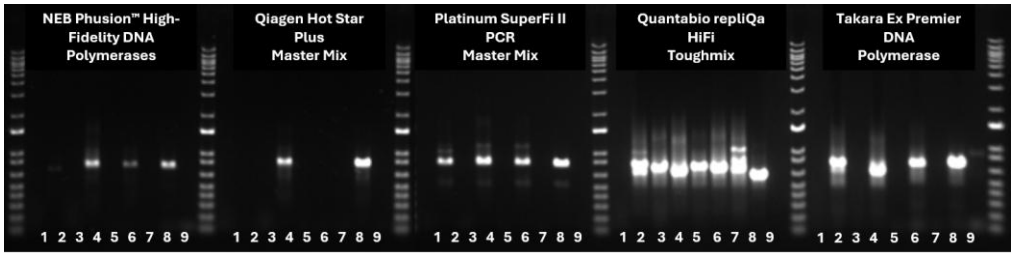


Figure 44. Amplification of PCR products (~800 bp) from air samples using primer set 1389f/ITS4ngs in combination with different PCR DNA polymerase mixes. All reactions were carried out using the manufacturer's instructions (see Appendix 5 for protocols). Samples: cyclone air sample sampled on 13/08/24 near arable field at Ebelsheerd (1), sample 1 purified through column (2), cyclone air sample sampled on 15/08/24 near arable field at Ebelsheerd (3), sample 3 purified through column (4), Pollensniffer sample from rose greenhouse sampled on 22/11/24 (5), Pollensniffer sample from gerbera greenhouse sampled on 16/05/24 (6), Pollensniffer sample from cucumber greenhouse sampled on 22/08/24 (7), positive control, DNA sample from the wheat pathogenic fungus *Zymoseptoria tritici* (8) & negative control, water (9).

The repliQa HiFi Toughmix seemed to be inhibitor resilient, showing strong amplification for all test samples, and was further used to amplify the fungal ITS1+2 target region of all selected greenhouse samples. However, no or

weak amplification was later still observed for a high number of selected rose samples and other rose samples were used to obtain a sufficient set of samples to be analysed by Nanopore sequencing. The ITS1+2 amplicons generated with primer set 1389f/ITS4ngs were sent to Leiden in a 96-well plate for Nanopore sequence analysis (Figure 45). Only the greenhouse samples were further analyzed as part of this project.

	1	2	3	4	5	6	7	8	9	10	11	12
A	G_4_01-1	G_4_13-62	G_4_23-138	G_4_43-266	R_5_06-38	R_5_22-94	R_5_44-176	R_5_61-253	C_6_08-29	C_6_16-62	C_6_24-92	V_2024_40
B	G_4_02-5	G_4_16-71	G_4_26-147	G_4_46-278	R_5_07-42	R_5_25-105	R_5_47-185	C_6_01-1	C_6_09-34	C_6_17-64	C_6_26-99	V_2024_45
C	G_4_03-7	G_4_17-97	G_4_29-159	G_4_50-327	R_5_08-45	R_5_26-108	R_5_49-192	C_6_02-6	C_6_10-36	C_6_18-69	C_6_27-104	L_2023_12
D	G_4_04-26	G_4_18-104	G_4_30-182	G_4_52-334	R_5_12-59	R_5_27-112	R_5_56-234	C_6_03-8	C_6_11-41	C_6_19-71	C_6_29-111	L_2023_29
E	G_4_06-36	G_4_19-111	G_4_34-196	G_4_56-348	R_5_13-64	R_5_28-115	R_5_57-239	C_6_04-13	C_6_12-43	C_6_20-76	C_6_30-113	L_2023_41
F	G_4_07-41	G_4_20-118	G_4_35-215	R_5_01-1	R_5_14-67	R_5_29-119	R_5_58-241	C_6_05-15	C_6_13-48	C_6_21-78	E_2024_07	P_290624
G	G_4_09-48	G_4_21-125	G_4_37-231	R_5_02-24	R_5_15-70	R_5_30-122	R_5_59-246	C_6_06-20	C_6_14-50	C_6_22-83	E_2024_10	IPO323
H	G_4_11-55	G_4_22-133	G_4_42-250	R_5_04-31	R_5_19-84	R_5_38-155	R_5_60-248	C_6_07-22	C_6_15-57	C_6_23-90	V_2024_33	Water

Figure 45. Plate with ITS1+2 amplicons generated with primer set 1389f/ITS4ngs. Samples starting with G, R and C are Pollensniffer samples from the gerbera (29), rose (28) and cucumber greenhouse (28), respectively. Last number indicates when sample was taken (the number of days after the start of sampling in each greenhouse). Samples starting with E and V are cyclone samples collected near arable fields at Ebelsheerd and Vredepeel, respectively. Samples starting with L and P are Rotorod samples collected near arable fields at Lelystad and in Polen, respectively. IPO323 is a DNA sample of *Zymoseptoria tritici* and last sample on the plate is a water control.

Metabarcode analysis of cucumber greenhouse air samples

Figure 46 shows the results of metabarcode analysis on the ITS1+2 PCR amplicons that were generated from DNA extracted from 28 air samples collected by a Pollensniffer in a cucumber greenhouse.

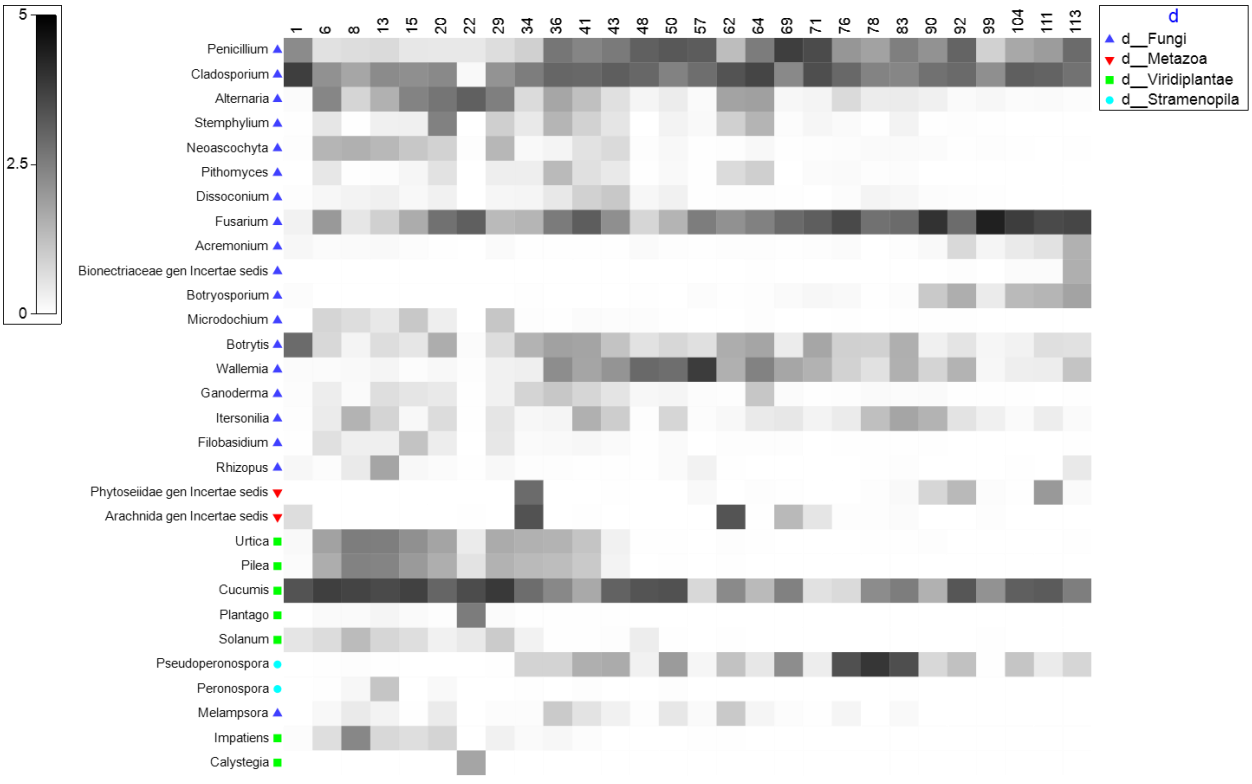


Figure 46. Long-term dynamics of fungal communities in air samples in a cucumber greenhouse using metabarcoding. The top 30 ITS1+2 PCR amplicon sequencing hits that were identified are displayed. Daily 24-h air samples were collected with a Pollensniffer during a whole crop cycle from 18/07/24 till 07/11/24, a 113-day period.

In addition to fungal sequences, ITS1+2 hits were also obtained from plants. As expected, high numbers of reads were recorded from cucumber as the crop itself, but also from plants belonging to the genus *Solanum*, to which tomato, potato and the eggplant (aubergine) belong. For *Pilea* and *Urtica* similar results are expected as they both

represent flowering plants in the nettle family *Urticacea*. The stinging nettle (*Urtica dioica*) flowers from April till October and the which coincides with the period July-August here where we see detection. The same is valid for the flowering plants belonging to the genus *Impatiens*, flowering from half June till end of September.

Regarding to the identification of plant pathogenic fungi and oomycetes, we grouped all genera detected according to their potential to cause plant diseases and/or having different lifestyles in Appendix 6. Fungi with allergenic properties and/or opportunistic human pathogens are listed in Appendix 7. Detection of the genus *Pseudoperenospora*, a group of oomycetes is likely due to the presence of *Pseudoperenospora cubensis*, an important cucumber pathogen causing downy mildew, being detected after 1 month till the end of the crop in the greenhouse (Figure 46). *Botrytis cinerea* was also detected, while *Fusarium oxysporum* f. sp. *radices-cucumerinum* associated with root and stem rot of cucumber might be part of the *Fusarium* genus that was detected. The cucumber pathogens *P. xanthii* and *M. cucumis*, although detected using qPCR, were not detected with metagenomics due to insufficient number of reads to belong to the top 30. Spores belonging to the genera *Penicillium*, *Cladosporium* and *Alternaria*, all well known airborne fungi with allergenic properties, were, as expected, detected in high numbers in most air samples. Detection of *Wallemia* spp. is a surprise, but *W. sebi* has been found in agricultural environments all around the world. With its relatively small-sized airborne spores (2.5-3.5 µm), easy to spread through air, it's being linked to human allergies, including bronchial asthma and farmer's lung disease (Zeng *et al*., 2004). Other fungi belonging to top 30 and being identified in multiple samples are representative of genera strongly associated species causing plant diseases (*Ganoderma, Itersonalia*, *Melampsora, Microdochium*, *Neoascochyta* and *Stemphylium*), living as weak (opportunistic) plant pathogens and/or as saprophytes (*Botryosporium* and *Rhizopus*) or being non-pathogenic, often with beneficial effects on plants (*Acremonium*, *Dissoconium*, *Filobasidium* and *Pithomyces*) (see Appendix 6).

Metabarcode analysis of gerbera greenhouse air samples

Figure 47 shows the results of metabarcode analysis on the ITS1+2 PCR amplicons that were generated from the 29 gerbera greenhouse DNA aerosol samples.

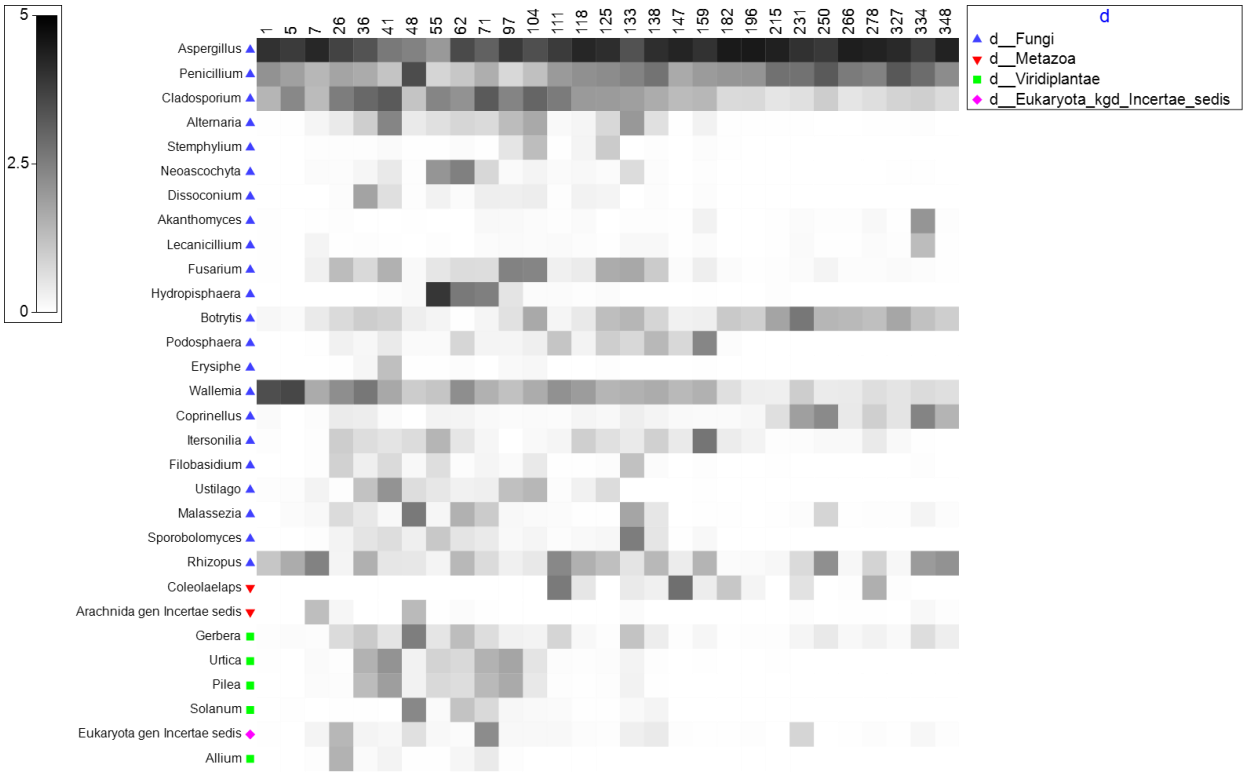


Figure 47. Long-term dynamics of fungal communities in air samples in a gerbera greenhouse using metabarcoding. The top 30 ITS1+2 PCR amplicon sequencing hits that were identified are displayed. Daily 24-h air samples were collected with a Pollensniffer for almost a year, from 16/5/24 till 29/4/25.

Regarding detection of plant pollen or other parts, a similar trend observed for the cucumber greenhouse was found for gerbera. Gerbera as crop was detected, as well as plants belonging to the genera of *Solanum*, *Urtica* and *Pilea*.

The gerbera pathogens *P. xanthii* (genus *Podosphaera*) and *B. cinerea* were both detected in the air samples and showed a similar dynamic over the sampling period as measured by targeted qPCR detection (see Figure 25). *Erysiphe*, a distinct genus under the powdery mildews was also detected at low numbers, but these spores can also be explained by mildew development and release of spores from other plants or trees outside the greenhouse. Low to high levels of fungal spores belonging to the genera *Alternaria*, *Cladosporium, Penicillium* and *Wallemia* were detected in all or most samples. In contrast to the cucumber greenhouse, where *Aspergillus* spp. was absent in the top 30, high read numbers for *Aspergillus* spp. were detected in gerbera greenhouse air samples during the whole sampling period. *Fusarium* spp. reads were detected for a shorter period. Other fungi making up the top 30 and being identified in multiple samples are representative of genera strongly associated species causing plant diseases (*Fusarium*, *Itersonalia*, *Neoascochyta*, *Stemphylium* and *Ustilago*), living as weak (opportunistic) plant pathogens and/or as saprophytes (*Rhizopus*) or being non-pathogenic, often with beneficial effects on plants (*Akanthomyces*, *Coprinellus*, *Dissoconium*, *Filobasidium*, *Hydropisphaera*, *Lecanicillium*, *Malassezia* and *Sporobolomyces*) (see Appendix 6). *Rhizopus* can occasionally show up as a secondary mold on dead, bruised, or already-decaying plant tissue (such as fallen flower petals or composted debris), but it does not actively infect healthy gerbera crops. As a necrotroph, *Fusarium* and *Itersonalia* spp. can cause petal spots and rotting to ornamental cut flowers like gerberas. Mushroom-forming *Coprinellus* species play a beneficial role in ecosystems by obtaining nutrients by decomposing dead and decaying organic matter. *Akanthomyces* are *Lecanicillium* are genera of insect parasitizing insects, often used for biocontrol, while species belonging to *Dissoconium* and *Hydropisphaera* live as mycoparasites. *Malassezia* represents lipid-dependent yeasts that live as commensals and opportunistic pathogens on the skin of humans and other warm-blooded animals. *Sporobolomyces* contains yeast species that can live on the surfaces of plants and have been used in biocontrol of plant pathogenic fungi. Some species belonging to the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Malassezia*, *Penicillium*, *Rhizopus* and *Wallemia* can cause allergic reactions, eczema and mycoses in humans who are susceptible and/or immunocompromised (Appendix 7).

Metabarcode analysis of rose greenhouse air samples

Figure 48 shows the results of metabarcode analysis on the ITS1+2 PCR amplicons that were generated from the 28 rose greenhouse DNA aerosol samples.

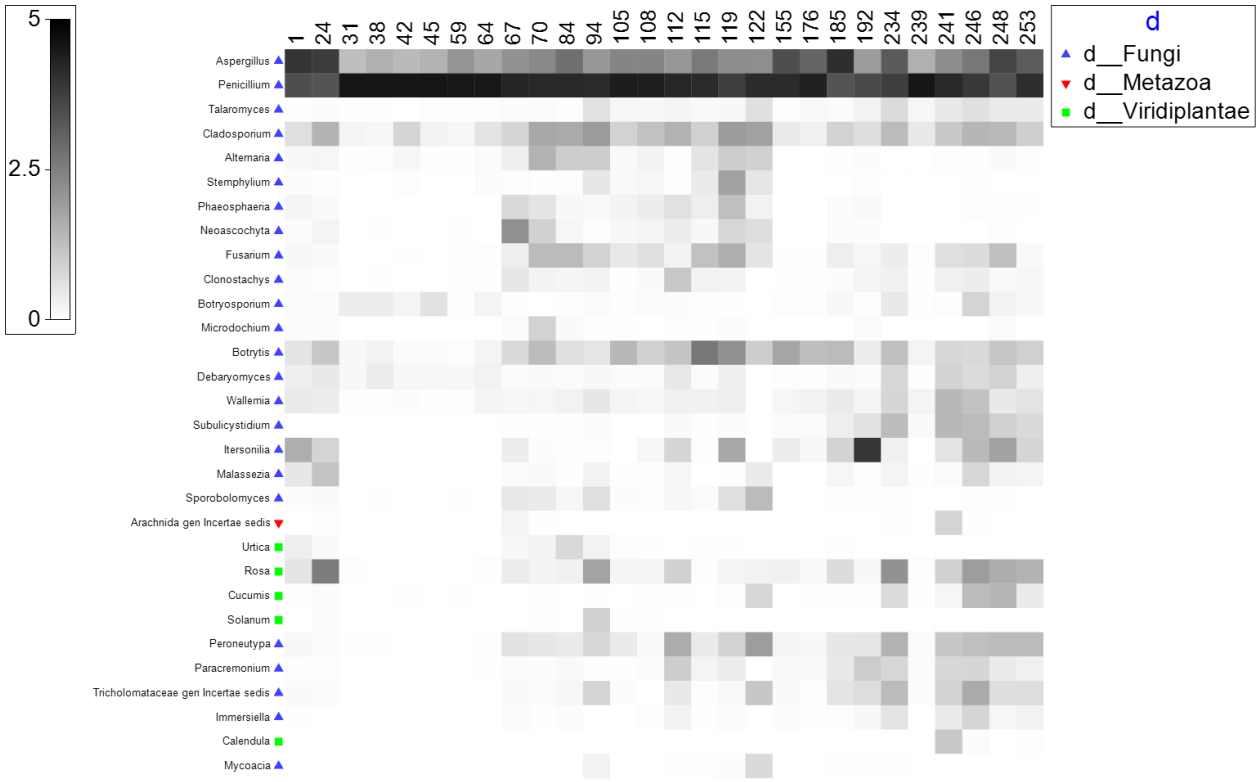


Figure 48. Long-term dynamics of fungal communities in air samples in a rose greenhouse using metabarcoding. The top 30 ITS1+2 PCR amplicon sequencing hits that were identified are displayed. Daily 24-h air samples were collected with a Pollensniffer during a 253-day period from 20/5/2024 till 28/1/2025.

Regarding detection of plant pollen, only low levels of *Urtica* reads were detected for short periods in aerosols of the rose greenhouse. As expected, rose DNA was detected in most samples while cucumber (*Cucumis*) was mainly detected in samples collected during January 2026, probably due to spread of pollen from nearby located greenhouses.

The pathogen *P. pannosa* causing powdery mildew in rose, being detected in qPCR, was not identified as a top 30 target in air samples. In comparison with the air samples from cucumber and gerbera greenhouses, high levels of *Penicillium* spp. were detected in the rose greenhouse aerosols, while lower levels of *Cladosporium*, *Aspergillus* and *Botrytis* were detected in all samples. Other fungi belonging to top 30 and being identified in multiple samples represent genera strongly associated with species causing plant diseases (*Alternaria*, *Fusarium*, *Itersonalia*, *Neoascochyta*, *Phaeosphaeria* and *Stemphylium*), living as weak (opportunistic) plant pathogens and/or as saprophytes (*Botryosporium, Paracremonium* and *Talaromyces*) or being non-pathogenic, often with beneficial effects on plants (*Clonostachys*, *Debaryomyces, Immersiela, Malassezia, Mycoacia*, *Peroneutypa*, *Sporobolomyces* and *Subulisystidium*) (see Appendix 6). *Paracremonium* spp. are primarily saprotrophs, but their diverse genus exhibits multiple lifestyles, including acting as opportunistic necrotrophic plant and human pathogens (Appendix 7). *Talaromyces* species are predominantly saprotrophs, but certain species can also act as necrotrophic plant pathogens or harmless plant endophytes. *Debaryomyces* are saprotrophic free-living, single-celled yeasts that thrive as harmless decomposers. *Immersiela*, *Mycoacia* and *Subulisystidium* are all fungi that can live as saprotrophs on decayed wood. *Peroneutypa spp*. are primarily classified as saprotrophs, but they are highly adaptable and frequently exhibit facultative necrotrophic or endophytic lifestyles

8. Discussion and conclusions

Selection and efficacy of air samplers for capturing bioaerosols in greenhouses. Different air samplers were tested for their ability to capture airborne plant pathogenic bacteria and fungi in greenhouses with cultivation of cucumbers, gerberas and roses. Based on air sampling volumes and the efficiency to capture both bacteria and fungal spores, affordability, availability and ease of use in operation and downstream sample processing, six different devices were selected for further studies after a literature search. To test air samples for the presence of plant pathogens using molecular tools, several DNA extraction methods were used and compared. In contrast to other extraction kits, DNA extracted from spiked and unspiked samples prepared with the MasterPure Yeast DNA Purification kit was highly suitable for sensitive detection of both bacteria and fungi using targeted (qPCR and LAMP) and semi-targeted detection (metabarcoding). Using a mixed inoculation of bacteria (*Pseudomonas amygdali* pv. *lachrymans*) and fungi (*Podosphaera xanthii* and *Botrytis cinerea*) in the air of an experimental glasshouse, the Coriolis Micro (wet cyclone) was highly efficient catching smaller particles (bacteria), while the MAS-100 (filter-based impaction), Rotorod (centrifugal rotating-arm impaction) and Pollensniffer (fan-operated suction) were also very effective in capturing larger particles (fungal spores). The Multi-Vial and High-Volume cyclone samplers that were tested were less efficient in capturing bacteria and fungi in the experimental glasshouse trial. Due to a focus on fungal pathogens and operational ease in continuous sampling and DNA extraction, the Rotorod and Pollensniffer devices were further used to collect air samples in glasshouse experiments and commercial greenhouses carried out by research staff and growers, respectively. Both devices were successfully employed to catch airborne fungal spores of powdery mildew (*Podosphaera xanthii* and *P. pannosa*), botrytis (*Botrytis cinerea*) and/or mycosphaerella (*Stagonosporopsis* spp.). Using qPCR assays targeting multi-copy DNA markers such as ITS, spores could be detected with both devices at a concentration of approximately one spore per m^3. The ultra-fast on-chip qPCR assays developed for detection of *B. cinerea* showed great potential, as results were comparable to qPCR chip and detection was achieved within 10 minutes.

Further studies revealed that air moves slowly in greenhouses. Wind speeds of 0.12, 0.16 and 0.21 m s^{-1} were measured by us in greenhouses cultivating roses, gerberas and cucumber, respectively. For cucumber powdery mildew, we established that spread of spores from inoculated plants could only be measured near infected plants. Therefore, multiple devices might be needed in a greenhouse to measure initial foci of disease. Detection can be more sensitive if larger volumes of air can be sampled with the Pollensniffer and Rotorod, currently being at a capacity of 10 and 37.5 L of air per minute, respectively. Devices can also be placed where disease is more likely to develop. Sokolidi *et al*. (2023) monitored the spread of tomato mildew (*Erysiphe neolycopersici*) in four commercial greenhouses throughout the growing season. After geostatistical modelling the patterns of infection, including the use of kriging, they found three forms of distribution. The first arose from numerous foci as spores landed on the leaves from various sources spread by air currents and the movement of workers along the paths through the greenhouse. The second resulted from an infection introduced through the main door in one corner of the greenhouse and spread from there by the workers and air currents, while the third infection arose near the center of the greenhouse by the main path and spread outwards from there. In all three examples, the main pathways were important for further spreading disease and this should be a key position to place air samplers. Sampling whilst moving, for example on a harvest car or picking vehicle, walking with a portable device, such as the Pollensniffer, or the use of a drone can also detect and/or map the distribution of spores earlier and allow growers to act earlier with disease intervention measures to prevent outbreaks.

Long-term targeted detection of pathogens in air samples from commercial greenhouses. Contrasting long-term dynamics were found for botrytis (*B. cinerea*), powdery mildew (*Podosphaera* spp.) and mycosphaerella (*S. citrulli*) in commercial greenhouses during cultivating of cucumbers, gerberas and roses using qPCR. In the rose greenhouse where roses were cultivated for the second year, medium levels of both *B. cinerea* (10^2-10^3 spores m^{-3}) and *P. pannosa* (10-10^2 spores m^{-3}) were continuously measured during a 253-day period (20/5/2024-28/1/2025). In the gerbera greenhouse, where a new crop of gerberas was growing, airborne spore levels were measured during a 348-day period (16/05/24-24/04/25). Levels of *B. cinerea* spores increased gradually from 10 spores m^{-3} in May to 10^4 spores m^{-3} at the end of November after which it dropped to a level of 10^2-10^3 spores m^{-3} till the end of the sampling period. Levels of *P. xanthii* increased gradually from 10 spores m^{-3} in May to 10^4 spores m^{-3} in October after which they dropped dramatically to very low levels of 1-10 spores m^{-3} during December till the end of April. The presence of airborne *B. cinerea*, *P. xanthii* and *S. citrulli* spores were also monitored in a cucumber greenhouse

for the whole crop cycle for 113 days (18/07/24-07/11/24). Levels of *B. cinerea* were quite constant in most air samples (10^2-10^3 spores m^{-3}). Levels of *P. xanthii* were initially very low (~1 spore m^{-3}) but increased gradually after one month when harvesting started to a level of 10^2-10^3 spores m^{-3} till the end of September after which the levels dropped to <10 spores m^{-3} till harvest. The drop in spores during the second part of the harvesting period might be due to a change in harvesting frequency from six to three times per week. Increased levels of *S. citrulli* spores were also detected after one month, when harvesting started, and stayed high till harvest. As climatical conditions in the greenhouses are kept constant (see Figure 49), arrival and spread of spores in the greenhouse will be affected by anthropogenic activities such as cleaning, removing leaves and flowers, harvesting and application of crop protection products.

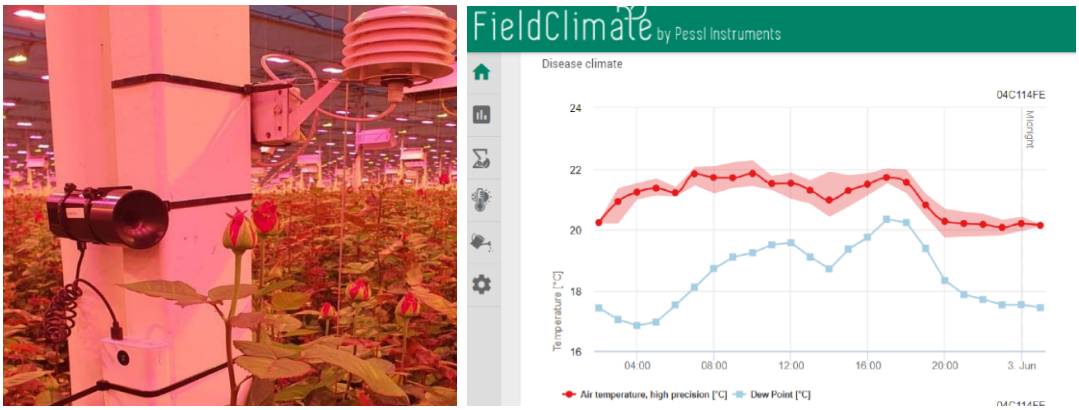


Figure 49. Measuring climate conditions in rose greenhouse using a wireless solar-powered Pessl Instrument Metos device (left, next to the Pollensniffer). The sensor measures air temperature and relative humidity and provides calculations for dew point, Vapor Pressure Deficit (VPD) and delta T (right).

A questionnaire (see Appendix 8) was sent but not much further info was obtained from the growers or information, such as treatments with crop protection products, needed to be kept confidential. Further studies should focus on one crop-disease combination where spore-trapping data in combination with disease forecasting can potentially directly guide the use of crop protection products (e.g. timing, choice and rate) as part of an integrated pest management, aiming to maximize control while minimizing environmental impact, human health risks, and the development of pesticide resistance. This study should establish how many sampling points are needed in a greenhouse and at which threshold (spores m^{-3}) intervention is needed to avoid a disease outbreak with an economic impact. It might also be possible to check if spores are alive or dead live/dead by combining standard qPCR with DNA-intercalating dyes (Vesper et al., 2008) or RNA detection using TaqMan-based RT-qPCR (Van der Steen *et al.*, 2022).

Metabarcoding studies on air samples from commercial greenhouses. DNA from air samples taken by Rotorod and Pollensniffer samplers were also successfully used in metabarcoding studies, revealing which bacterial and fungal genera are specifically or omni-present in different greenhouses. The technology can be further improved by improving DNA extraction and purification, as some DNA samples, especially sampled in the rose greenhouse showed inhibition during amplification of long PCR products, designing new primers or adding new primers to increase the number of different bacterial and fungal species that can be detected and identified, and to improve the bioinformatic analysis pipeline by removing and/or adding in new DNA barcodes. The benefit of applying metabarcoding was demonstrated by its ability to detect and identify the presence of multiple plant pathogens, saprophytic and/or beneficial microorganisms as well as (opportunistic) pathogens for plants, insects and humans in air samples. The commercial cucumber grower, who collected air samples during the whole crop cycle, reported to have sprayed fungicides twice against downy mildew. The presence of the causal pathogen *Pseudoperonospora cubensis* was confirmed by high read numbers for *Pseudoperonospora*. The highest numbers of reads were measured two weeks after the second spray. Although airborne spores of *Stagonosporopsis citrulli* (aka *M. cucumis* or *D. bryoniae*) were detected by qPCR, this pathogen didn't belong to the top 30 species with the most detected reads (Figure 46). The proportion of DNA of this pathogen might be relatively low in comparison with the other targets, but it is also possible that the number of reads might be underestimated due to low specificity of the primers used in metagenomics. For example, we noticed that DNA from the barley net blotch pathogen *Pyrenophora teres* is not amplified with the ITS1+2 targeting primer set. There were some differences in the mycobiome composition associated with the different greenhouses over time. For example, the highest number of

reads in the rose and gerbera greenhouse were measured for *Penicillium* and *Aspergillus* spp., respectively, while high read numbers of *Cladosporium* spp. and *Fusarium* spp. were detected in the cucumber greenhouse. In addition to these genera, high read numbers of *Botrytis* spp. and *Wallemia* spp. were also frequently found in all greenhouses. The presence of these fungi in greenhouse aerosols has also been confirmed in other studies (e.g., Li & LaMondia, 2009; Madsen *et al*., 2021; MonsÓ *et al*., 2002). Further studies using metabarcoding will be needed to check if the different greenhouse mycobiomes, or at least part of it, found in this study are more a reflection of the crop itself or a consequence of the outdoor environment in combination with the environmental conditions in the greenhouse itself. Metabarcoding can also be applied to evaluate the effect of green crop protection products, such as application of antagonists and their direct and indirect impact on the plant's ecosystems. For example, whether an added antagonist survives, colonizes and/or shifts the desired microbial balance on or in the plant, and if the overall long-term resilience and health of the plant improve.

New developments in combining spore trapping and detection. Further improvements in detection are expected with the introduction and application of automatic spore trap devices like the Swisens Poleno Jupiter and the BioScout which both rely on machine learning. The Swisens Poleno Jupiter is an automated, real-time bioaerosol monitoring instrument that continuously draws in ambient air to capture holographic images, light scattering, and UV-induced fluorescence signals of individual airborne particles, using artificial intelligence to identify and count spores. The Swisens Poleno Jupiter has been used to recognize spores of different *Alternaria* and *Cladosporium* spp. with mixed success (Bruffaerts *et al*., 2025). The fully autonomous BioScout spore trap (Figure 50) works by continuously sampling the air and using automated optical imaging (400x microscopy) combined with artificial intelligence (AI) to detect, identify, and count airborne fungal spores in real time.

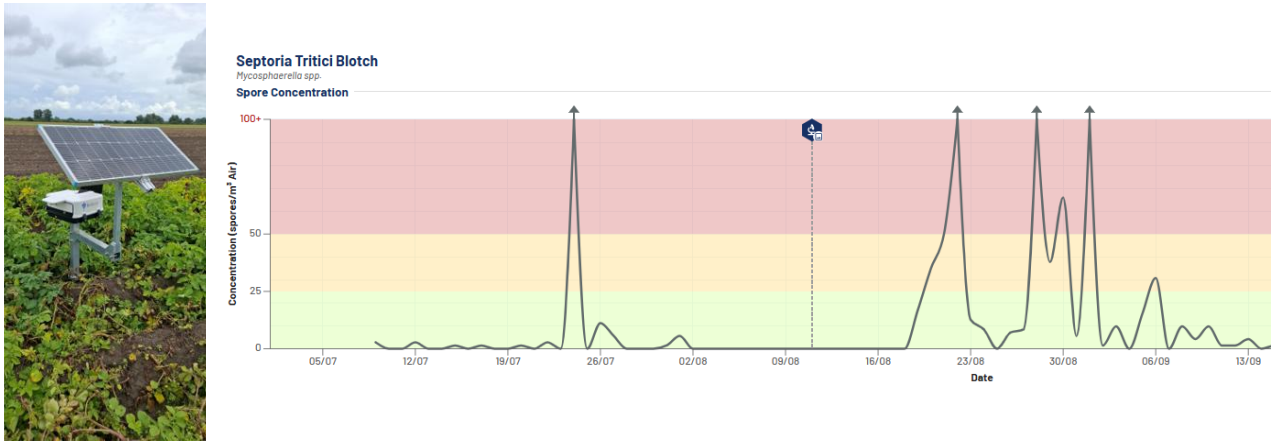


Figure 50. The BioScout spore trap positioned in a potato field for monitoring airborne sporangia of the potato pathogen *Phytophthora infestans* (left) and results of daily ascospore monitoring of *Zymoseptoria tritici* (causal agent of Septoria tritici blotch in wheat) near Lelystad (the Netherlands) during late summer in 2026 (right).

AI models for the BioScout have already been developed to recognize ascospores of *Mycosphaerella* and *Sclerotinia*, conidiospores of *Alternaria*, *Botrytis*, *Erysiphe* and *Stemphylium*, sporangia of *Peronospora* and *Phytophthora*, and urediniospores of *Puccinia*. Like the Swisens Poleno Jupiter, identification on the species level might be difficult to achieve as it will depend on the difference in morphology of the spores. However, the *Mycosphaerella* ascospore model might also work for *Stagonosporopsis citrulli* (aka *Mycosphaerella cucumis*) as the ascospores of *Zymoseptoria tritici* (aka *Mycosphaerella graminicola*) are morphologically very similar. As data will be collected continuously, the BioScout could be very useful in determining which activities in the greenhouse contribute to disease outbreaks and under which climatical conditions in the greenhouse (e.g., humidity and temperature) the production and spread of spores of key pathogens can be restricted while maintaining the quantity and quality of produce. The methodology developed during this project, combining air sampling with DNA-based detection, will be very useful to validate the results of automatic spore trap devices as this will enable confirmation of the presence of specific species. For example, if mycosphaerella and botrytis spores are detected in a cucumber greenhouse, are these all or mainly derived from spores spreading from foci caused by *M. cucumis* (*S. citrulli*) and *B. cinerea*, respectively? As samples cannot be retrieved from the currently available automatic spore trap devices to carry out pathogen isolation or DNA testing, other air sampling devices need to be employed alongside them. Portable air sampling devices, such as the Coriolis Compact and the Pollensniffer, can also be used for extra sampling as soon as positive results are obtained with an automatic spore trap device. Samples from these

devices can also be used for metabarcoding and on-site testing using LAMP assays as demonstrated for detection of mycosphaerella in cucumber fruits in this study. As mycosphaerella can be caused by three different *Stagonosporopsis* spp., we confirmed the presence of *S. citrulli* in a separate PCR reaction. A LAMP assay for a direct and specific detection of *S. citrulli* in plant tissues has recently been reported by Pineda *et al*. (2026). On-site LAMP assays can also be used to test for important traits of fungal pathogens like fungicide resistance as shown for *Botrytis cinerea* (Hu *et al*., 2017), *Didymella bryoniae* (Shen et al., 2022) and *Podosphaera xanthii* (Vielba-Fernández et al., 2019).

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APPENDICES

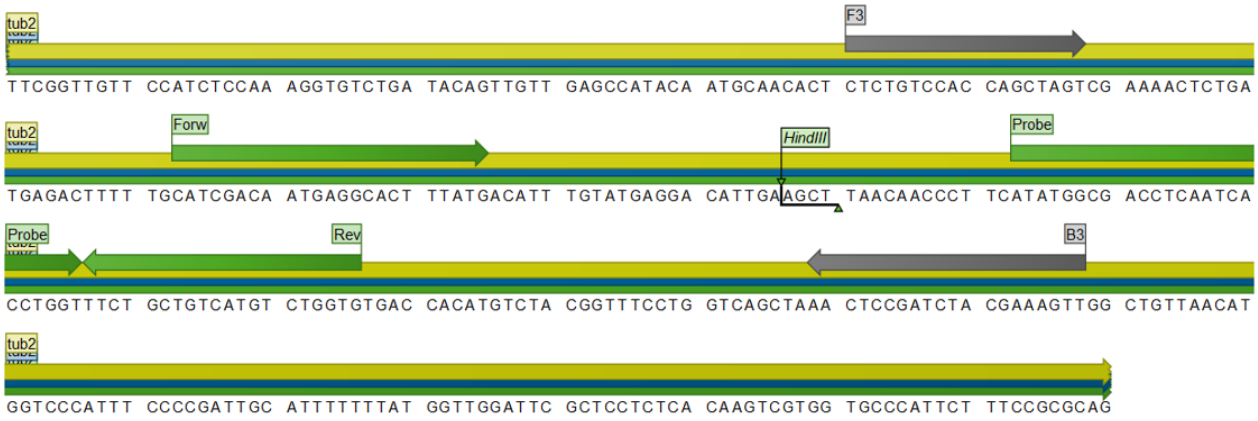
Appendix 1. gBlock sequences for detection of target DNA

CTAAGAACATGATGGCCGCTTCCGATTTCCGTAACGGTCGTTACTTGACATGCTCTGCCATTT*TG*
*TAAGTTTGCCCTGTAATCAATCTGCCAAAAT*CTTGTAGAAACTAACTTTCTGTAGCCGTGGTAAG
GTTTCCATGAAGGAGGTTGAGGACCAAATGCGCAACGTCCAAAACAAGAACTCATCCTACTTCGT
TGA (198 bp)

gBlock DNA sequence targeting beta-tubulin from *Botrytis cinerea* with primers (forward 5'-GTTACTTGACATGCTCTGCCATT-3' and reverse 5'-CACGGCTACAGAAAGTTAGTTTCTACAA-3') underlined and probe shown in italics (5'-ATTTTGGCAGATTGATTACAGGGCAAACTTACA-3')

ACCTGCGGAAGGATCATTACAGAGTTCATGCCCGAAAGGGTAGACCTCCACCCTTGTGTATTATT
ACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGAGAAAACCAAAACTCTTT
TTATTAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAA
CCCTCAAGCTTAGCTTGGTATTGAGTCTATGTCAGTAATGGCAGGCTCTAAAATCAGTGGCGGCG
CCGCTGGGTCCTGAACGTAGTAATATCTCTCGT*TACAGGTTCTCGGTGTGCTTCTGC*CAAAACCC
AAATTTTCTATGGTTGACCTCGGATCAGGTAGGGATACCCGCTGA

gBlock DNA sequence (500 bp) targeting the ITS region from *Botrytis cinerea* with primers (forward 5'-GTCAGTAATGGCAGGCTCTAA-3' and reverse 5'-GATCCGAGGTCAACCATAGAAA-3') underlined and probe shown in italics (5'- TACAGGTTCTCGGTGTGCTTCTGC-3')



gBlock targeting β-tubulin from *Podosphaera xanthii* with positions of the primers (forward 5'- CATCGACAATGAGGCACTTTATG-3' and reverse 5'-ATATGGCGACCTCAATCACCTGGT-3') and probe (5'-CACCAGACATGACAGCAGAA-3') all indicated. Length of gblock is 350 bp.

CTAGGGAGAGTGGCAACGCTCTCCCAGGGCGGAAAGTTGTCCAAACTTGGTCATTTAGAGGAAGT
AAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAGCGCGAGGCCCCG
CAGCGCGCACGCGCTGCGGCGGTTGACCCTCCACCCGTGTGAACTCTTATCTGTTGCTTTGGCGG
GCCGGGCTCGACCTGCCGGCTCCGGCTGGCGAGTGCCCGTCAGAGAAGCCCCAACT*CGTGCTGTG*
*AGTGTTGTCTGAGGAA*ATGTGGAATTAGTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATC
GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTTAGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCCCCGGCATTCCGAGGGGCATGCCTGTTCGAGCGTCAGAACACCCCT
CAAGCCTAGCTTGGTCTTGGGGCTCGCCGGCTCGG

gBlock targeting the small subunit ribosomal RNA gene from *Podosphaera xanthii* with primers (forw. 5′-CCACCCGTGTGAACTCTTATC-3′ and rev. 5′- CATTTCGCTGCGTTCTTCATC-3′) underlined and probe (5′-CGTGCTGTGAGTGTTGTCTGAGGAA-3′) in italics. Length of gblock is 490 bp.

TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAGCGCGAAGCCACGCAGGACGCTTGTCCTGCG
CGGCTGACCCTCCACCCGTGTGAACTGAATTTGTTGCTTTGGCGGGCCGGGCTCGACCTACCGGC
TCCGGCTGGGGAGTGCCCGCCAGAGAAGCC*CCAACTCGTGCAGTTAGTGCAGTCT*GAGAAAAACT
TAATAAGTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTTAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCC
CGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCAGAACATCCTCTCAAGCCTGGCTTGGTCTTGG
GGCGCGCCGGCTCGGCGTCCCCTAAACAGAGTGGCGGTACCGTTGTGCTCTCCGCGTAGTCACGT
ATCTCGCGACAGAGTGGCGACGGGACCTGCCAAAACCCACCTAT

gBlock targeting the small subunit ribosomal RNA gene from *Podosphaera pannosa* with primers (forw. 5′-CACCCGTGTGAACTGAATTTG-3′ and rev. 5′-GAGCCAAGAGTCCGTTGTT-3′) underlined and probe (5′-CCAACTCGTGCAGTTAGTGCAGTCT-3′) shown in italics. Length of gblock is 499 bp.

TGGGCAGTCAATCCTTATCCTGTGCAGGATAGGCAGATCGAATGGCTCAGAGAAGGTCACTCTGG
GACACCCGAGACCAAGAGGCTACACGACGAGGATGCAGCTGACGAGTCTGAGCGGAAAGAGTACA
ACCTGCTCAACTGCGATGGATATATCAACATGACTGAGATTGTCAAGGTTCGTAAGGCAAGACTA
GGCGCAACTCCAGCGGATGAAGACATCGAGGATGGGTCTGACGTGGACTTTGACGAGGATGTTGA
CATGGATGG*GTCCAGAGATGAGGATGGAGTCA**CGAAGGATATTGATCTAGACCGCACTTTC*GAGC
TAATCATGAAAAATGGGCTCATTATTCGCCTACAAGCCGCAGACCAGGCGCGCCGAAAGGAATGG
ATCAAACATCTCAGGGCCCTCACAAAGTACTGGAGACATCGCACCGCATCCGACATCACCCTTTA
CAAGTCCACAAGGTCCCGCAATCTCAGCGCCCTAAACATCGATGAGGAAGGCGAAGCACACATTG
GCCAGTTCGCCCGCAAATGGGAGGTCACACAATCTTTCG

gBlock targeting a RAPD DNA fragment from *Stagonosporopsis citrulli* (aka *M. cucumis*) with primers For. 5′- GTCCAGAGATGAGGATGGAGT-3′ and Rev. 5′-GGCTCATTATTCGCCTACAAGC-3′) underlined and probe (5′- CGAAGGATATTGATCTAGACCGCACTTTC-3′) shown in italics. Length of gblock is 559 bp.

Appendix 2. DNA extraction from air samples using the Epicentre MasterPure Yeast (MPY) DNA Purification Kit

Materials needed

The following materials are not included in the kit:

70% Ethanol

Iso-propanol

1.5 mL or 2 mL micro-centrifuge tubes

A 1:1:1 (w:w:w) mix of glass beads (sizes: <106 μm, 425-600 μm and 710-1180 μm)

3.175 mm stainless steel beads (Vanem)

2 mL Bertin Precellys bead-beat tubes (o-ring tube)

Step 0

Samples collected using air samplers should be stored and processed in 2 mL Bertin Precellys bead-beat tubes.

Comment: if processing samples containing a Tape sample be sure that sticky side is placed towards center and not against the wall of the tube.

Step 1

Add approximately 100 mg of glass beads and optional one 3.175 mm steel bead to each sample collected in step 0.

Comment: The optimal size and combination of beads depend on matrix and organism.

Add 300 μl of Yeast Cell Lysis Solution to each bead-beat tube containing sample.

The optional RNAse treatment is skipped for air samples.

Bead-beating

Bead-beat all samples using the Bertin Precellys bead-beater with the following protocol:

6000 rpm, 3 steps of 10 seconds with a 15 second pause between steps.

Step 2

Incubate the suspended cells at 65° C for 15 minutes at 750 rpm.

Step 3

Place the samples on ice for 5 minutes.

Add 150 μl of MPC Protein Precipitation Reagent and vortex mix for 10 seconds.

Comment: if processing samples containing a Rotorod sample rod it may be best to remove the plastic rod during this step. Leaving the rod in the sample will lead to disturbing the debris pellet after the next step.

Step 4

Pellet cellular debris by centrifugation in a microcentrifuge for 10 minutes at $\geq$10,000 rpm (approximately 11,000 g).

Step 5

Transfer the supernatant to a clean microcentrifuge tube and add 500 μl of isopropanol (not provided).

Mix thoroughly by inversion.

Step 6

Pellet the DNA by centrifugation in a microcentrifuge for 10 minutes at ≥10,000 rpm (approximately 11,000 g).

Step 7

Remove the supernatant by pipetting and discard.

Wash the pellet containing the DNA with 500 µl of 70% ethanol by centrifugation for 30 seconds at ≥10,000 rpm.

Carefully remove the ethanol by pipetting and discard.

Briefly centrifuge the DNA pellet and remove any remaining ethanol.

Air-dry the DNA approximately 15 minutes so all traces of ethanol have evaporated.

Step 8

Suspend the DNA in 35 µl of TE Buffer.

Store the DNA at 4° C.

Appendix 3. PCR reactions targeting 16S and ITS2 carried out using the Thermo Scientific™ Phire Hot Start II DNA polymerase enzyme

# Reactions +1 =	91		Marker:	16S	

PCR reactie mix:					
merk	Chemical	Conc.	reaction (µL)	mastermix (µL)	
	NF water		16.58	1508.78	O
	Phire Green HS	2x	23.68	2154.88	O
	primer F	10 µM	2.37	215.67	O
	Primer R	10µM	2.37	215.67	O
	Template	~ 25 ng/µl	5		
Total			50µl*	4095	

** voor optimalisatie PCRs gebruik je 20 µl. Voor sequence PCR's schaal je op naar 50 µl*

PCR program:					
		Temp.		Time	
Initial denature	1x	98	°C	0:00:30	
Denature	35x*	98	°C	0:00:05	
Annealing		51	°C	0:00:05	
Extension		72	°C	0:00:25	
Final Extension	1x	72	°C	0:01:00	
Pause		15	°C	∞	

** voor metabarcoding PCR's start altijd met 30 cycli en verhoog indien nodig*

# Reactions +1 =	91		Marker:	ITS2	

PCR reactie mix:					
merk	Chemical	Conc.	reaction (µL)	mastermix (µL)	
	NF water		16.58	1508.78	O
	Phire Green HS	2x	23.68	2154.88	O
	primer F	10 µM	2.37	215.67	O
	Primer R	10µM	2.37	215.67	O
	Template	~ 25 ng/µl	5		
Total			50µl*	4095	

** voor optimalisatie PCRs gebruik je 20 µl. Voor sequence PCR's schaal je op naar 50 µl*

PCR program:					
		Temp.		Time	
Initial denature	1x	98	°C	0:00:30	
Denature	40x*	98	°C	0:00:05	
Annealing		50	°C	0:00:05	
Extension		72	°C	0:00:15	
Final Extension	1x	72	°C	0:01:00	
Pause		15	°C	∞	

Protocols for amplification of bacterial 16S using primers 27F and 1492R (top) (91 and fungal ITS2 using primers ITS3 and ITS4, each for 91 reactions

Appendix 4. Fungal species identified using ITS2 sequence analysis



Appendix 5. PCR reactions to amplify ITS1+2 DNA fragments from fungi. Quantabio repliQa HiFi Toughmix

The following reagents were mixed to carry out 25 µl PCR reactions using the Quantabio repliQa HiFi Toughmix. Primers 1389F (5'- TTGTACACACCGCCC-3') and ITS4ngs (5'- TCCTSCGCTTATTGATATGC-3') were used to amplify ITS1+2 fragment of approximately 800 bp from fungal DNA.

Quantabio repliQa HiFi Toughmix							
						1rxn	
Step	**Temperature (˚C)**		**Time**		**Component**	**µl**	
					repliQa HiFi Toughmix (2X)	12.5	1x
Denature	98	35x	10 sec		F primer (10µM)	1.25	500nM
Anneal	55		5 sec		R primer (10µM)	1.25	500nM
Extend	68		5 sec		MilliQ	9	
Hold	4		oneindig				
					target	1	

Appendix 6. Genera of fungi and associations with plants

Genus	Genera of fungi and oomycetes to which species belong that are strongly associated with plant pathogenicity
Alternaria	*Alternaria* is a cosmopolitan genus of fungi containing many plant pathogens that exhibit a necrotrophic lifestyle.
Botrytis	A genus with highly destructive necrotrophic plant pathogens, affecting a wide range of agricultural crops, ornamental plants and wild plant species
Didymella	Generally classified as necrotrophic or hemi-biotrophic plant pathogens, rather than obligate biotrophs
Fusarium	A genus that represents major plant pathogens, found in soil and cause devastating diseases in a wide variety of economically important crops.
Ganoderma	Species can function as both plant pathogens and saprotrophs, as facultative parasites or hemi-biotrophs that switch between living and dead wood
Itersonilia	*Itersonilia* act as both plant pathogens and saprotrophs. As a necrotroph, it can cause petal spots and rotting to ornamental cut flowers like gerberas
Melampsora	A group of fungi commonly known as rust fungi, affecting trees and crops. They are obligate biotrophic parasites, requiring living plant tissue to survive.
Microdochium	Many species are necrotrophic plant pathogens; others function as saprotrophs or endophytes (living inside plants without causing disease)
Neoascochyta	This genus includes a wide variety of plant-pathogenic, saprotrophic, and endophytic fungi
Peronospora	Fungus-like oomycetes (commonly known as water moulds) that cause downy mildew, a severe disease affecting various crops
Phaeosphaeria	*Phaeosphaeria* spp. have different lifestyles, living as necrotrophic pathogens, saprotrophs or endophytes
Plectosphaerella	Many species are well-known necrotrophic plant pathogens, though the genus also contains soil-borne or endophytic strains
Podosphaera	These fungi are obligate biotrophic parasites, meaning they require a living plant host to survive and reproduce
Pseudoperonospora	Fungus-like oomycetes that cause downy mildew, a severe disease affecting various crops. Closely related to *Peronospora*.
Stemphylium	While many species act as saprotrophs or endophytes, several species are aggressive necrotrophic plant pathogens affecting dozens of plants
Ustilago	True plant pathogens, commonly known as the smut fungi, primarily infecting plants in the grass family (*Poaceae*), including cereals

Genus	Genera of fungi with species often showing weak phytopathogenic properties and/or a saprotrophic lifestyle
Aspergillus	*Aspergillus* represents several important plant pathogens, though its species are primarily recognized as opportunistic pathogens or saprotrophs
Botryosporium	*Botryosporium* includes species capable of acting as weak or opportunistic plant pathogens, though many species primarily function as saprotrophs
Cladosporium	While majority of species within this genus act as environmental saprotrophs, some species are biotrophic pathogens
Paracremonium	Primarily saprotrophs, but their diverse genus exhibits multiple lifestyles, including acting as opportunistic necrotrophic plant and human pathogens
Penicillium	Mostly saprophytes, but certain species cause destructive post-harvest diseases in crops, particularly fruits, entering plants through wounds or bruises
Rhizopus	*Rhizopus* is a genus that includes plant pathogens, although its members are primarily known as saprotrophic moulds living on decaying organic matter.
Talaromyces	*Talaromyces* spp. are predominantly saprotrophs, but certain species can also act as necrotrophic plant pathogens or harmless plant endophytes

Genus	Genera of fungi harboring non-phytopathogenic species with epiphytic, saprotrophic and/or endophytic lifestyles
Acremonium	Represents a group of filamentous molds that are widely distributed in nature, primarily acting as saprotrophs in soil and dead organic matter
Agaricus	The genus *Agaricus,* which includes the common button mushroom *Agaricus bisporus*, consists of terrestrial saprotrophic fungi
Akanthomyces	Primarily entomopathogens, belonging to the family *Cordycipitaceae*, which means they specialize in parasitizing insects, spiders, and mites
Beauveria	*Beauveria* can live as endophytes and are widely known as entomopathogenic fungi. *Beauveria bassiana* is used as biological insecticide
Clonostachys	*Clonostachys* spp. are saprotrophs, often endophytic, and are widely recognized as biological control agents that fight against plant diseases
Coprinellus	Mushroom-forming *Coprinellus* species play a beneficial role in ecosystems by obtaining nutrients by decomposing dead and decaying organic matter
Debaryomyces	*Debaryomyces* are saprotrophic free-living, single-celled yeasts that thrive as harmless decomposers
Dioszegia	Orange-to-red pigmented non-pathogenic basidiomycetous yeasts that naturally inhabit the phyllosphere as well as oligotrophic soils
Dissoconium	*Dissoconium species are* largely recognized for their biotrophic relationship with other fungi as mycoparasites
Filobasidium	*Filobasidium* are basidiomycete yeasts frequently isolated from plants, the phyllosphere and seeds. They are generally harmless or even beneficial
Hydropisphaera	A genus primarily consists of saprophytic or fungicolous species, surviving by breaking down decaying organic plant matter or living as mycoparasites
Immersiela	They live as saprotrophs, growing on dead organic matter such as decaying wood, plant debris, or dung in terrestrial ecosystems
Lecanicillium	A genus primarily representing entomopathogenic fungi. Used as biocontrol agents against insects
Malassezia	Lipid-dependent yeasts that live as commensals and opportunistic pathogens on the skin of humans and other warm-blooded animals
Mycoacia	They are saprotrophs, wood-decay fungi that break down dead organic matter, causing white rot on fallen logs and dead branches of broadleaved trees
Peroneutypa	Primarily classified as saprotrophs, but they are highly adaptable and frequently exhibit facultative necrotrophic or endophytic lifestyles
Pithomyces	Classified as saprobes, living on dead or decaying organic matter, such as the leaves, stems, and litter of a wide variety of plants and grasses
Sporobolomyces	A genus of saprophytic, pink-colored yeasts that live harmlessly on the surfaces of plants
Subulicystidium	*Subulicystidium* species are saprotrophs, growing on decayed wood, fallen logs, dead bark, and other plant debris on the forest floor
Trametes	Some species can act as opportunistic plant pathogens or weak parasites, though most are primarily saprotrophic wood-decay fungi
Wallemia	Found on seeds, straw, hay and grains, they are saprotrophic spoilers rather than pathogens and thrive in environments with very low water availability

Appendix 7. Genera of fungi with impact on humans

Genera	**Common genera of fungi with human pathogenic and/or allergic properties**
Alternaria	One of the most potent outdoor and indoor aeroallergens worldwide; strongly linked to severe asthma (e.g., *A. alternata*)
Aspergillus	Ubiquitous environmental molds causing allergic bronchopulmonary aspergillosis, chronic pulmonary infections, and invasive aspergillosis in severely immunocompromised patients (e.g*., A. fumigatus*)
Cladosporium	A very common outdoor mold that frequently causes allergic rhinitis and asthma (e.g., *C. herbarum*)
Fusarium	*Fusarium* species can trigger allergic rhinitis (hay fever), asthma, and allergic fungal sinusitis, as well as cause Fusariosis in immunocompromised patients (e.g., F*. solani*)
Malassezia	Yeast skin commensals linked closely to the exacerbation of atopic dermatitis (eczema), particularly around the head and neck
Paracremonium	Can cause subcutaneous infections (phaeohyphomycosis), localized subcutaneous nodules, slow-growing abscesses or cysts (e.g., *P. inflatum*)
Penicillium	Primarily indoor moulds thriving in damp areas, water-damaged buildings, and spoiling food, contributing to year-round respiratory allergies (e.g., *P. chrysogenum*)
Rhizopus	Can cause allergic reactions, affecting the upper and lower respiratory tracts, and occasionally mucormycosis in severely immunocompromised individuals (e.g., *R. stolonifer*)
Wallemia	Inhaled spores impact on human health primarily through respiratory allergies, asthma exacerbation, and hypersensitivity pneumonitis (e.g. *W. sebi*)

Appendix 8. Questionnaire to obtain meta-data

Lucht bemonstering apparatuur questionaire	
Algemene informatie	Antwoorden
Naam of teler (code wordt later gebruikt)	
Plaats/lokatie	
Datum	
Tuinbouwkas volume (L x B x H) (m^3)	
Type bemonsteringsapparatuur	
Positie in kas	
Hoogte van luchtbemonstering	
Bemonsteringsperiode/tijdstip	
Bemonsteringsduur	
Bemonsteringsnelheid (m^3 lucht/minuut)	
Bemonsteringsvolume (m^3)	
Monster	
Lucht temperatuur	
Relatieve luchtvochtigheid (%)	
Lokale luchtstroom (m^3 lucht/minuut))	
Ventilatie (verticaal/horizontaal)	
CO_2 concentratie	
Specifieke informatie	
Gewas (indien meerdere geef % van elk gewas)	
Ras	
Startdatum van het gewas/plantdatum	
Waarschijnlijke oogstdatum	
Groeistadium en/of ouderdom van gewassen	
Is er een ziekte aanwezig?	
Welke ziekten?	
Frequentie van zieke planten of aantal plaatsen per kas oppervlakte	
Belichtingsduur (fotoperiod)	
Lichtspectrum	
Lichtintensiteit	
Schermdoek (type en merk)	

Tuinbouwkas activiteitenlijst (geef ook aan hoe lang de activeit duurt per dag)	Dag 1	Dag 2
Snoeien van planten		
Bladplukken		
Verwijdering plant materiaal		
Plukken van bloemen		
Vruchtdunning		
Pesticide toepassingen (welk middel en hoe toegepast)		
Oogst van gewas/vrucht		
Verwisselen van gewas		
Schoonmaak/onderhoud		
Andere activiteiten met invloed op luchtstroom of beweging van planten		