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Volatile organic compounds in greenhouse: Discovery of volatile biomarkers related to fungal infection by *Botrytis cinerea* in tomato plants

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Abstract

The development of fungal disease in greenhouse plants can result in limited production and great economic losses for the producer. Early detection and identification of disease in plants before visible symptoms appear, could contribute to a more effective and sustainable production for Norwegian growers. Cucumber (*Cucumis sativus* L.) and tomato (*Solanum lycopersicum* L.) are common and important plants produced in Norwegian greenhouses. *Pythium* sp. root rot and grey mould caused by *Botrytis cinerea* infection are some of the most severe plant diseases, and particularly connected to cucumber and tomato plants. Both diseases can be difficult to notice by visual observation and are easily transmitted within a crop.

Current research on volatile organic compounds (VOCs) is mainly focused on plant response to herbivores and fruit quality, while the research related to pathogens is more limited. If fungal infection causes a release of unique VOCs specific to the disease, these can be detected by highly sensitive analytical method HS-SPME-GC-MS. This is a non-invasive method that can be used to test for specific disease biomarkers without harming of the plants.

Here we show that a large number of VOCs can be identified by this method. The sesquiterpenes α-copaene and cis-thujopsene are classified as candidate biomarkers for fungal infection in tomato plants, and possibly also for the specific interaction of *B. cinerea* related disease. The monoterpenes β-phellandrene and (+)-4-carene were also identified as important compounds released in high concentrations, likely associated with general stress response in tomato plants and tomato-related smell components.

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Abbreviations

ANOVA	Analysis of variance
BP	Base peak
DAI	Day(s) after infection
GC	Gas chromatograph
GC-MS	Gas chromatography coupled to mass spectrometry
GLV	Green leaf volatile
HS	Headspace
MS	Mass spectrometer
MSD	Mass selective detector
MW	Molecular weight
m/z	Mass to charge ratio
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PDA	Potato dextrose agar
PLS-DA	Partial least squares discriminant analysis
ppm	Parts per million (10^{-6})
ppt	Parts per trillion (10^{-12})
RI	Retention index
RT	Retention time (min)
SIM	Selective ion monitoring
SPME	Solid phase microextraction
TIC	Total ion chromatogram
VIP	Variable importance projection
VOC	Volatile organic compound

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1 Introduction

1.1 Background

Tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.) are some of the most important crops related to Norwegian greenhouse production. 40 % of cucumber production and 85 % of tomato production on a national basis, is located in the county of Rogaland. This makes Rogaland, and especially the region of Jæren, the most significant part of Norway when it comes to agriculture (NIBIO, 2021).

During commercial production of tomato and cucumber in greenhouses, crop infections caused by fungal disease are one of the most limiting factors. Infection by these pathogenic organisms can result in severely reduced production yields and economic losses for the producer. According to Petrasch et al. (2019), in a worldwide perspective, 20-25 % of all harvested fruit and vegetables are lost due to rotting caused by fungal pathogens (Petrasch et al., 2019). The health status of greenhouse crops is primarily assessed by human inspection including visual symptom observation, and various approaches have been made to prevent the development of disease in tomato and cucumber plants. This includes the use of tolerant or resistant cultivars and development of fungicides. However, only a few resistant cultivars are available on the market, and the use of fungicides offer the potential concern of resistance and also environmental contaminations (Islam et al., 2019).

Gray mould is a disease caused by the fungal pathogen *Botrytis cinerea,* which can cause infection in a wide range of plants including tomato. All parts of the plant can be affected by the fungi, but symptoms on the leaf and stem is most common. Characteristic traits of the disease are necrotic spots containing fuzzy dark coloured sporulation. In humid conditions and low temperatures, the spores are easily released to infect adjacent plants (Hermansen et al., 2018). The pathogen can remain dormant in the host tissue for long periods of time, which in many cases makes it difficult to notice visual symptoms of the fungal disease (Gao et al., 2018).

Root rot caused by *Pythium* spp. is a common disease for greenhouse produced plants, and one of the most severe when it comes to cucumbers. The fungi thrive in conditions of high temperatures and relative humidity, and spreads easily throughout the crop by producing

zoospores which travel in the damp soil. It can also be transmitted by water droplets, insects and infected tools (Hermansen et al., 2011). Visual characteristics of infection by *Pythium* is usually a brown rot at the roots and core of the stem. Growth may be stunted, in addition to wilting of the lower leaves (Sankaranarayanan & Amaresan, 2020).

Similarly for both *Botrytis* grey mould and *Pythium* root rot, when infection is confirmed on a plant or crop it is already severe and not easily dealt with. Usually, a large part of the crop will need to be disposed of to prevent further spreading of the disease. In order to minimise crop losses caused by fungal pathogens, early-stage detection and identification can be essential to control the spread of infection. This would in turn result in maximised productivity and agricultural sustainability (Fang & Ramasamy, 2015). Plants constantly emit volatile organic compounds (VOCs), which might vary between healthy and diseased plants. Profiling of the volatile signatures released from infected plants can be achieved by GC-MS analysis even before visual symptoms appear. This is a non-invasive method that can be used to test for specific disease biomarkers without harming of the plants (Ray et al., 2017). One of the main challenges is the identification of specific volatile biomarkers which are unique for certain plant-pathogen interactions, and also differ from volatiles produced by general stress or environmental conditions. Earlier research on VOCs is mainly focused on fruit quality and plant responses to herbivores, while the investigation of pathogen-related emissions is currently limited (López-Gresa et al., 2017).

A biological marker, or biomarker, is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (Mandal, 2021). Plants react to various environmental conditions or stressors, ranging from internal biochemical processes to the development of symptoms caused by disease or otherwise inflicted damage. Volatile organic metabolites can be identified as possible biomarkers for disease detection. Biomarkers may be specific for a single compound, a group of compounds, or as an indication of general stress applied on the plant. Different stress factors can result in the same impact on a plant’s metabolism (Ernst, 1999).

1.2 Volatile organic compounds in plant research

1.2.1 Definition of volatiles

Volatile organic compounds, also called VOCs, are compounds of low molecular weight, high vapour pressure at room temperature, and low polarity. This includes a variety of gases that are released into the air from solid or liquid sources. VOCs have high volatility and will easily evaporate at room temperature, and they have the ability to diffuse though the air and soil (Petrisor & Paica, 2019). Plant-emitted volatile organic compounds are secondary metabolites with defensive functions towards pathogens and herbivores (López-Gresa et al., 2017). Many types of VOCs are produced by plants, and the largest groups consist of terpenes and green leaf volatiles (GLVs). Both groups contribute to a range of different odours, which by humans may be perceived as fruit related aromas (Buettner, 2017).

1.2.2 Why and when plants emit VOCs

Living organisms produce a large number of volatile organic compounds as a part of their metabolic processes. This includes plants, which emit VOCs into their surroundings to serve essential functions in growth, communication, defence and survival. VOCs released from leaf surfaces are metabolites than can give indications to the physiological health status of the plant (Martinelli et al., 2015). Plant leaves normally release small amounts of volatile compounds, but this can be increased by damage on the plant. The type of molecule depends on the plant species and the type of herbivore or pathogen attack. Unharmed plants will keep a baseline level of volatile metabolites that are emitted from the surface and from accumulated storage sites in the leaves. These chemical reserves often include monoterpenes, sesquiterpenes and aromatics, which accumulate to high levels in specialised glands or trichomes (Paré & Tumlinson, 1999).

Studies show complex interactions between plants and various stresses, with increased VOCs as a host response. Biotic stress factors have a biological cause, and in greenhouse production that generally include herbivore infestation and pathogen infection of the plants. The responses to abiotic stress are normally caused by environmental influences which are non-biological

(Jansen et al., 2010). These include temperature, light, the deficiency of water or nutrients, and variation in CO_2 or O_2 levels.

The composition of emitted volatile compounds may also be dependent on factors such as developmental stage of the particular plant strain, type of stressor, damage, and the severity and timespan of the stress (Kasal, 2016; Petrisor & Paica, 2019). The increase of certain substances can happen at an early stage or directly after stress has been applied to the plant, while the onset of other emissions can start to reveal itself after several hours or days. This might give an indication of which type of stressor the plant has been exposed to (Jansen et al., 2010).

1.2.3 Emission of plant-related VOCs in greenhouse

Many sources of VOCs will exist inside of a greenhouse environment. The specific crop inside of the greenhouse will likely be the most important source of VOCs in this space. But due to greenhouse ventilation, which is the removal of air from the greenhouse and replacing it with air from the outside, the gas balance of plant volatiles might be affected by a transfer of volatiles from external sources. The ventilation aspect is likely the most important factor to affect the composition of VOCs, though there might be other loss processes happening. Other loss processes include degradation due to gas-phase reactions, sorption on surfaces such as the floor of the greenhouse, solution of VOCs in water bodies on cold surfaces due to condensation, absorption on the plant cuticle, and uptake of volatile compounds through absorption in the stomata of plants. A slow loss of VOCs in a greenhouse would cause an accumulation and thus promote detection. A fast loss would on the other hand enable the detection of short time volatile dynamics (Jansen et al., 2010).

It was suggested that measuring the types and amounts of different terpenoids in the air can be a way to monitor the health of crops in greenhouses. Especially in closed greenhouses with no free ventilation the volatiles would be able to accumulate to make detection more achievable (Thelen et al., 2005). Methyl salicylate is a volatile compound that commonly can be detected because of biotic stress to the plants, but the compound may also be found in greenhouse due to emissions from the ripe tomato fruits. This means that it might be best used as a marker for general stress in leaves of the tomato plant (Kasal, 2016).

Crop operations in greenhouse such as harvesting fruits and removal of side shoots, as well as fluctuations in temperature, has been observed to affect the emissions of volatile monoterpenes and sesquiterpenes (Jansen, Hofstee, Wildt, Verstappen, Bouwmeester, & van Henten, 2009). With climate control in greenhouses it would usually make it possible to avoid great changes in temperature and light conditions, which would generally contribute to an increased rate of emitted VOCs (Jansen et al., 2010). To measure the plant VOCs in a greenhouse, one would have to first collect the plant-emitted volatiles, separate the compounds in the mixture, and lastly identify and/or quantify the different separated VOCs.

Jansen et al. (2009) investigated the correlation between damage of trichomes by stroking of tomato plants, and the following increase of terpene concentrations, in greenhouse conditions. For stress-related compounds such as α-copaene, methyl salicylate and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, concentrations were not significantly affected by trichome damage. The monoterpene β-phellandrene and sesquiterpene β-caryophyllene, on the other hand, showed large increases in concentration after the same treatment (Jansen, Hofstee, Wildt, Verstappen, Bouwmeester, Posthumus, et al., 2009).

1.2.4 Relevant VOCs for plant-pathogen response

Generally, plant-related VOCs include of a wide range of different chemical structures, such as terpenoids, phenylpropanoids, benzenoids, fatty acids and amino acids. Non-stressed plants also emit volatiles, which in tomato plants originate from epidermal structures or glandular trichomes on the plant surface. The trichomes will contain monoterpenes such as 2-carene, α-phellandrene, α-terpinene, limonene, β-terpinene and β-phellandrene, and sesquiterpenes such as β-caryophyllene, α-humulene and δ-elemene. Monoterpenes and sesquiterpenes are the source of the typical smell in tomato fruits and leaves (Kasal, 2016).

According to Jansen et al. (2010), after a stress-induced change in VOC emission in plants, independent of type of stressor and plant species, some frequently reported chemical substances include (Z)-3-hexenol, methyl salicylate, (E)- β-ocimene, linalool, (E)- β-farnesene, (E)-4,8-dimethylnona-1,3,7, triene, and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Jansen et al., 2010).

In addition to the mentioned plant volatiles, an inoculation of fungal pathogens will lead to a larger total set of volatiles. This is due to the production of VOCs by fungi or fungi-plant interactions. The volatile organic compounds can act as plant defence compounds and be emitted as direct resistance agents or resistance inducing signals on the fungal phytopathogens (Quintana-Rodriguez et al., 2018). The specific VOCs will largely depend on the type of fungi used in infection of the plant, and the plant itself.

Thelen et al. (2005) suggests that the sesquiterpene α-copaene is a significantly distinctive component associated with fungal infection in tomato leaves, and possibly connected to the infection caused by *B. cinerea*. After GC-MS analysis of the volatile components emitted, mostly mono- and sesquiterpenes were detected (Thelen et al., 2005). Kasal (2016) also mentions α-copaene as a relevant compound in the infection of tomato plants by *B. cinerea,* and she proposes that it is stress-induced and likely plays a role in the plant's defence against pathogens (Kasal, 2016). Some compounds can be released from plants when exposed to infection, as an antifungal property. Sánchez-Ortiz et. al points out 3-methyl-1-butanol and thujopsene as such compounds, after inoculation of tomato plants and other plants with various fungal pathogens such as *Xylaria* sp. "Pure thujopsene isolated from *Thujopsis dolabrata* presents antibacterial activity […] thujopsene also acted as a self-regulation mechanism against the growth of *Penicillium decumbens*" (Sánchez-Ortiz et al., 2016). This might give an indication of thujopsene being a relevant compound in connection to plant responses against fungal infection.

1.3 Analysis of VOCs

1.3.1 Headspace sampling (HS)

The most suitable approach for collecting biological material from plants depends on the chemical composition of the compounds of interest. According to Michael A. Birkett (2010), the most common technique for volatile organic compounds, and especially those of odour or aroma characteristics, is collection by headspace (Baluška & Ninkovic, 2010). Headspace sampling is a separation technique which makes it possible to extract volatile material from

heavy and complex sample matrices and inject it into a gas chromatograph for analysis (Tipler, 2020).

Within a sealed and heated vial, the heavy molecules will remain in the liquid phase while molecules of a higher volatility will migrate into the headspace phase. A small volume of the headspace vapour from a vial is transferred manually, or automatically by the headspace sampling system, into the GC column. This will make the chromatography cleaner, easier and faster due to a far lower amount of the less-volatile material entering the GC column (Tipler, 2020). For headspace sampling in vials, the equilibration time can be decreased by increasing the temperature and shaking the vial while extracting the volatiles.

Static headspace sampling is the simplest headspace analysis technique in principle. The sample is contained in a closed system, typically a vial, and can provide an indication of the aroma composition that can be detected by the human nose. Some advantages include few steps of sample preparation and no use of solvents. Though the sample should contain high amounts of volatile material for this analysis (Tromelin et al., 2006). Dynamic headspace sampling is based on a purging of the headspace with a large volume of gas which continually moves the headspace of the sample to concentrate the analytes onto a trap. Analytes remain on this trap until desorption on the GC, followed by separation and detection (Teledyne Tekmar, 2012).

1.3.2 Solid phase microextraction (SPME)

In 1990, Arthur and Pawliszyn introduced solid phase microextraction as an advanced extraction technique, and an effective alternative to liquid-liquid extraction (Arthur & Pawliszyn, 1990). SPME is a technique used to extract and concentrate compounds from headspace vapour. It is most suitable to the types of sample where the compounds of interest migrate first into the headspace and sorb onto the coating of the chosen fibre (Tipler, 2020). The SPME technique is an efficient and popular analytical tool for assessing the content of VOCs in quantities ranging from ppt to ppm. Due to the combination of extraction, concentration and introduction into one step, a reduction of preparation time can be achieved (Petrisor & Paica, 2019). SPME can offer increased sensitivity compared to other extraction methods, depending on the type of fibre that is applied (Tipler, 2020). The fibre can be chosen depending on its affinity for compounds of low or high molecular weight, or a combination.

Headspace sampling by SPME is performed by inserting a fibre into the sample vial containing headspace vapour. The fibre is located inside the needle of a special syringe and equipped with a retentive coating which is used as a “trap” for the volatile compounds. The SPME needle punctures the seal of the vial before extending the fibre down into the headspace to absorb or adsorb volatile molecules from the vapour (Tipler, 2020). When the sample material is located off-site from the instrumental setup, the analytes might be at risk of degradation by heat or oxygen during transfer. By transporting the sample material on a SPME fibre, the risk of degradation decreases as analytes are adsorbed onto the retentive coating. This can result in the collection of more accurate and precise analytical data.

After collection of headspace analytes onto a SPME fibre, it is moved to a heated GC inlet for desorption. This process is normally operated by an autosampler but can also be performed manually. Inside the inlet the fibre absorbs heat from the injector liner, which in turn desorbs the extracted analytes (Tipler, 2020). After the volatile compounds are released using heat, the analytes will be transferred through the GC column by a carrier gas for analysis. When performing an extraction by SPME, a splitless injection into the GC is required to focus analytes on the chromatographic column. According to Sigma-Aldrich, the splitter vent valve should be closed for at least two minutes during sample injection (Sigma-Aldrich Co., 2004).

1.3.3 Basic principles of GC-MS

Separation and detection of volatile metabolites are commonly obtained by gas chromatography coupled to mass spectrometry (GC-MS). Chromatographic methods are powerful separation methods used in many branches of science, including research, clinical analysis, the pharmaceutical industry and petroleum industry. It allows for separation, identification and determination of the components of complex samples (Skoog et al., 2017). In gas chromatography (GC), a sample is evaporated and carried by a mobile phase which is then forced through a stationary phase. The mobile phase is an inert gas, often helium or nitrogen, and the stationary phase a column where the sample compounds are separated with respect to size, polarity and other chemical-physical properties depending on the column characteristics (Petrisor & Paica, 2019). Retention times and volume are both used for the qualitative identification of compounds, while peak heights and areas give quantitative

information. The separation capabilities of GC can be combined with the identification properties of other instruments such as mass spectrometers to enhance qualitative identification of components (Skoog et al., 2017).

Constituents of complex mixtures of VOCs can be determined by flame ionization detection (FID), or most widely used mass spectrometry (MS). MS is a powerful technique used to identify and measure a wide variety of compounds. Basically, the mass spectrometer converts the sampled compounds into gaseous ions, and the most common ionization process for gas phase analysis involves bombardment of molecules with electrons by electron impact ionization (EI). The molecule is given enough energy to eject one of its electrons and become positively charged. The bombardment of electrons also results in fragmentation of the molecule, which gives a number of ions with different mass-to-charge (m/z) ratios. The fragmentation of each molecule is unique and is used as a chemical fingerprint to characterize the compounds. Then, compounds are identified using libraries or databases of mass spectra, or by comparison of retention time and spectra with those of authentic standards (Petrisor & Paica, 2019). MS is in general the preferred method for the profiling of metabolites in biological materials (Piasecka et al., 2019)

The instrumental method GC-MS provides powerful separation and highly sensitive detection capabilities, and is the analytical instrument of choice when it comes to VOC profiling (Martinelli et al., 2015). Coupled to HS-SPME it is able to show excellent detection levels allowing the identification of large numbers of VOCs in plant tissues and other biological matrices, including cucumber and tomato fruits. The gas phase separation and detection system is able to give extensive information on structural and chemical properties of the individual components (Cortina et al., 2017).

1.4 Objectives

The main objective for this research project was to investigate the VOCs released during infection of greenhouse plants by fungal pathogens. Based on this, four sub objectives were formulated:

I. Establishment of analysis for VOCs emitted by pathogen infected fruits in laboratory conditions

The first objective of the study was to establish an analytical method for the analysis of VOCs produced by *P. aphanidermatum* infected cucumber fruits and *B. cinerea* infected tomato fruits. This included optimisation of growth parameters for fungal cultivation, choosing the specific pathogen isolates used for inoculation of plant tissues in following experiments, and optimisation of analytical parameters.

II. Identification of VOCs released from cucumber plants infected with *P. aphanidermatum* using static sampling

The second objective was to identify specific VOCs related to the plant-pathogen response of *Pythium* sp. root rot in cucumber plants. A static sampling method was applied for collection of VOCs in the headspace of the plants.

III. Identification of VOCs released from tomato plants infected with *B. cinerea* using dynamic sampling

The third objective was to identify specific VOCs related to the plant-pathogen response of grey mould disease in tomato plants. A dynamic sampling system was set up for an improved collection of headspace volatiles. Possible biomarker candidate compounds were compared by the characteristics of compounds detected by GC-MS analysis of *B. cinerea* infected tomato fruits, to find out if volatiles were related to general stress responses or specific to the pathogenic interaction on the plants.

IV. Targeted analysis of VOCs produced by tomato plants infected by *B. cinerea*, in conditions more similar to those of a greenhouse

To find out if the biomarker candidates from ongoing *B. cinerea* infection in tomato plants could be detected in a larger volume of air more similar to greenhouse conditions, a GC-MS

analysis including selected ion monitoring (SIM) was performed on samples collected from the ventilation shaft of a growth room containing the diseased plants.

2 Materials and methods

2.1 Development of methodology

A literature review was performed to research the parameters of SPME and GC-MS, used in the analysis of tomato and cucumber related volatiles. A short review of the positive and negatives sides of different sampling and injection techniques was also performed, for which outcome can be found in the beginning of the results chapter. Their analytical function and sensitivity are important due to the low concentrations of specific VOCs which can occur in a greenhouse setting with monitoring of plant-related diseases.

2.2 Experimental design

Three experiments were conducted during this project. Due to the extent of preparation for the two plant experiments (E2 and E3), the first experiment (E1) was performed mainly as a qualitative test to confirm the level of infection by the chosen pathogens on similar tissues as the respective plants. Collections of headspace gases were also performed to look for pathogen characteristic volatile compounds in the different samples, to be compared with volatile compounds in the next two experiments.

During E1, visual examinations confirmed infection by *Pythium aphanidermatum* on cucumber, and infection by *Botrytis cinerea* on tomato. These observations led to the assumption of similar responses when infecting tomato and cucumber plants in the following two experiments.

Experimental sites for the project include the Norwegian Institute of Bioeconomy (NIBIO) in Særheim and the University of Stavanger. Preparations and practical experiments on plants were carried out in growth chambers and labs at NIBIO, while analysis was performed in the instrument lab at the University.

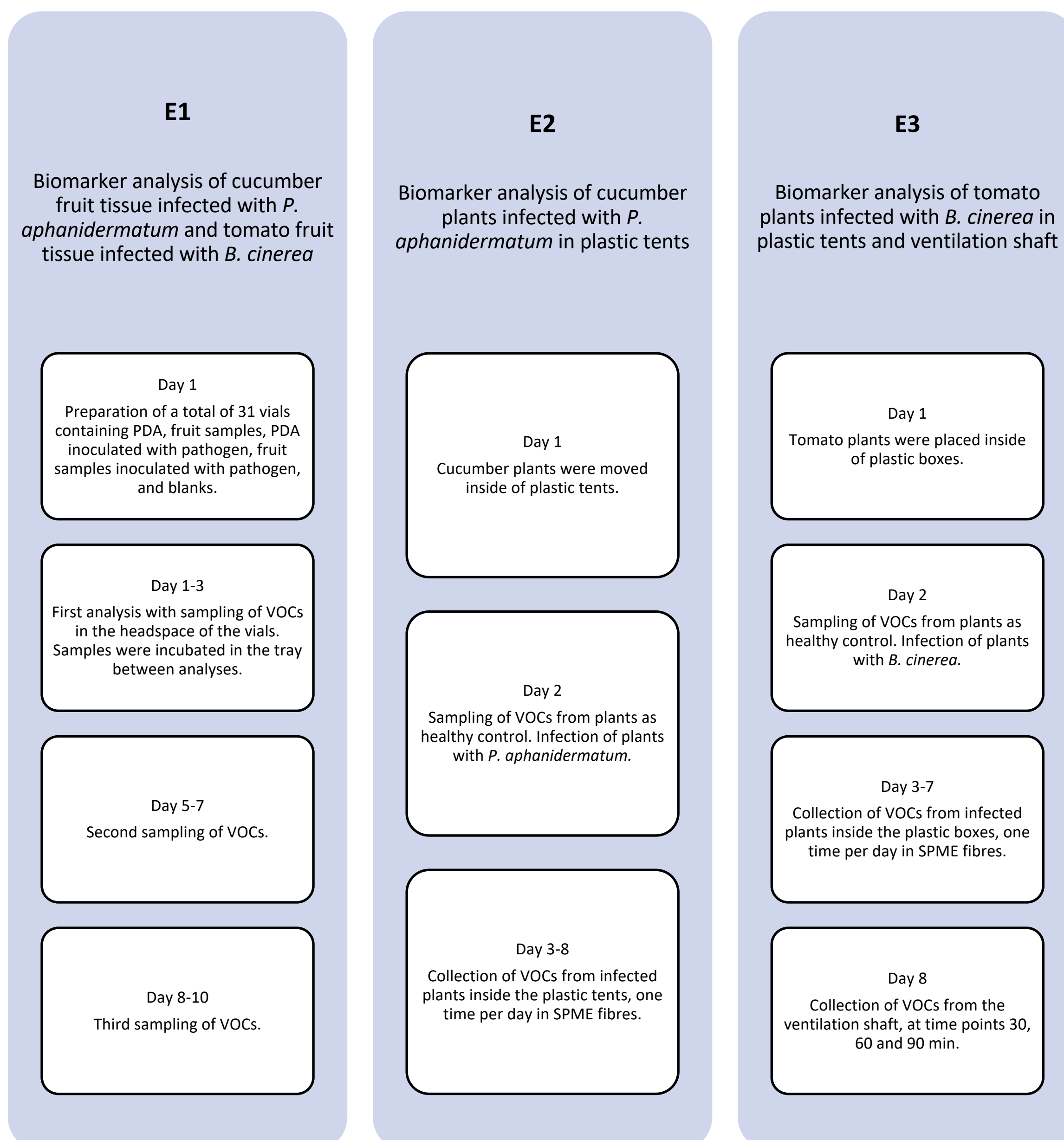


Figure 1: Flow chart of the three performed experiments E1, E2 and E3.

2.3 Biological materials

2.3.1 Plant materials and growth conditions

Experiments were performed on fresh cucumbers and tomatoes in E1, as well as cucumber and tomato plants in E2 and E3. Cucumbers were obtained from a local producer from Jæren region, while tomatoes were purchased from a grocery store and also produced locally.

Cucumber plants of the variety Topvision were sown in a rockwool substrate by a local producer on the 18th of January. The plants were delivered to the growth chamber at NIBIO 23 days later, on the 10th of February, and moved into pots the following day. Each of the substrates containing one cucumber plant were planted into 2-3 litre pots of peat, which was watered regularly with fertiliser water. By attaching the plants to ropes hung on a line across the room, it allowed them to grow upwards. Ropes were wrapped around the main stem of the plants, connected to a hook that was hung onto the horizontal line. Side shoots were removed, and the plants were kept in a growth chamber with an approximate temperature of 20 °C. The climate data was measured for growth room 12 which contained the cucumber plants, in an eight-day period during the experiment, from the 19th to 26th of February 2021. The room measured a temperature in the range between 19.5 and 20.5 °C, with a decrease of two degrees Celsius at night between 11 PM and 4 AM, and a relative humidity between 40 and 65 %. The plants also underwent an extermination procedure upon arrival in order to eliminate possible thrips infestation.

Figures 2A and B: Cucumber plants at the day they were delivered to NIBIO, repotted and fixed to ropes in order to grow in an upwards direction.

Tomato seeds were planted separately in small rockwool cubes on the 5th of February, covered with sand and watered. 17 days later the seedlings were transferred into larger cubes of 9.5 cm^2. The substrate was kept constantly moist with fertiliser water. The plants were maintained in a separate growth chamber with an approximate temperature of 21 °C. The climate data was

measured for room 11 which contained the tomato plants, in an eight-day period during the experiment, from the 3rd to 10th of March 2021. The room measured a temperature between 20.5 and 21.5 °C, and a relative humidity between 20 and 60 %.

Figures 3A and B: Tomato plants at 6 and 28 days of growth, before and after transfer to larger cubes of rockwool.

Table 1: Overview of plant materials used for the three experiments.

Plant materials	**Details**
Cucumber	Delivered from local producer in Jæren region
Tomato	Store bought, produced locally in Orre
Cucumber plants	Topvision variety, sown 18.01.2021
Tomato plants	Forticia RZ variety, sown 05.02.2021 at NIBIO

2.3.2 Pathogen cultures

Three different species of the fungal pathogen *Pythium* and four isolates of *Botrytis cinerea* were obtained from other NIBIO departments (more detailed information is listed in Table 2). These were all maintained on potato dextrose agar (PDA, Eur. Pharm., prod. nr. 84651.0500). Subculturing was performed inside a sterile cabinet (LAF bench) that was cleaned out with 70 % EtOH before and after use. For the transfer of *Pythium* spp. to new growth medium, a sterile scalpel was used to cut a small piece of agar containing part of the fungi, which was placed in the middle of a new plate of PDA with the mycelial side down. Plates were sealed with Parafilm and incubated upside down at 25-30 °C. After a couple of days, new fungal mycelium could be observed on the agar plates. Subculturing of both *Pythium* spp. and *B. cinerea* isolates was performed every 3-6 days by application of this method.

A test of pathogenicity was performed on the three species of *Pythium* and four isolates of *Botrytis cinerea* to decide which ones to use in the next experiments on plants. To sterilise the surface of the fruits, large pieces of cucumber were cleaned in a twice diluted chlorine solution containing some drops of Triton X-100 and rinsed thoroughly. For inoculation a small square slice was cut out of the cucumbers, a piece of agar containing *P. aphanidermatum, P. irregulare* and *P. dissotocum/coloratum* was placed in separate cucumbers, and the slice of cucumber was placed back on top. The inoculated cucumbers were then placed into separate autoclaved glass jars. A sheet of absorbent material and some drops of water had already been placed in the base of each jar to create a humid environment to aid in the development of fungi. Incubation was carried out in a dark growth room at 20 °C. Small pieces of cucumber were also cleaned and inoculated for control purposes. These were placed in 50 ml Falcon tubes and incubated in the same conditions as described above.

The jars and tubes of *Pythium* infected cucumber were examined after three and five days and based on visual observations, the most and fastest growing fungal species was chosen. The four single-spore isolates of *Botrytis cinerea* were cultured from conidia on cotton swabs and monitored as they developed on PDA. After growing the cultures for nine days and subculturing the fungi once, one isolate was chosen based on the same characteristics as for *Pythium* spp. above.

Table 2: Detailed information on the acquired pathogens. The two fungal pathogens chosen for further testing on cucumber and tomato plants are marked by an asterisk (*).

Species	Isolate	Host plant	Collector and location
*Pythium aphanidermatum**	231-099	Cucumber	NIBIO, Ås
Pythium irregulare	231-660	Cucumber	NIBIO, Ås
Pythium dissotocum or P. coloratum	231-641	Salad	NIBIO, Ås
*Botrytis cinerea sensu stricto**	96/19-2.8	Strawberry	NIBIO (Gunn Mari Strømeng), Grimstad
Botrytis cinerea sensu stricto	96/16-8.2	Strawberry	NIBIO (Gunn Mari Strømeng), Valle
Botrytis cinerea sensu stricto	Bc 101	Strawberry	NIBIO (Linda G. Hjeljord), Grimstad
Botrytis cinerea sensu stricto	Pa7	Norway spruce	NIBIO (Gunn Mari Strømeng), Biri

2.4 Inoculation, disease development and collection of VOCs

2.4.1 Experiment 1

The preparation of this experiment was performed in a sterile cabinet cleaned with 70 % EtOH before use, and all equipment introduced into the cabinet was also sterilised. Cucumber and tomato fruits were cleaned as described in chapter 2.3.2, and cut into small pieces. 29 screw-capped headspace vials of 20 ml with a magnetic cap and silicone septum (Supelco by Sigma-Aldrich, Germany) were assembled in the sterile cabinet and loaded with fruit and pathogen tissues as explained below in Table 3. The *P. aphanidermatum* plates used for this was from passage number five, and *B. cinerea* from passage number two. Twelve of the vials were already filled with liquid PDA to cover the bottom, which had solidified and cooled beforehand. It was strived to fill all vials with approximately the same sample volume, not exceeding 30 % of the vial height to avoid touching of the SPME fibre while sampling of VOCs (see Figures 13A and B on page 38).

Vials were transported to the instrument lab at UiS the same day and arranged in the incubation tray of the GC. Incubation then proceeded in the tray for the following ten days of analysis. The incubation temperature in the tray was measured to stay in the range between 26-29 °C during these days.

Table 3: Vial setup with more detailed information on the sample preparation procedures.

Number of vials	**Setup**	**Label**
4	One layer of tomato fruit pieces	T1-T4
4	Two layers of tomato with agar pieces of *B. cinerea* in between	TBC1-TBC4
4	One layer of PDA and agar pieces of *B. cinerea* at the top	ABC1-ABC4
4	One layer of cucumber fruit pieces	C1-C4
4	Two layers of cucumber with agar pieces of *P. aphanidermatum* in between	CPA1-CPA4
4	One layer of PDA and agar pieces of *P. aphanidermatum* at the top	APA1-APA4
4	One layer of PDA	A1-A4
1	Air sample as blank	B

The collection of volatiles by headspace solid-phase microextraction from sample vials was performed by a 2 cm 50/30 µm DVB/CAR/PDMS fibre (Supelco by Sigma-Aldrich, Germany) from the incubation tray of a 6890N GC (Agilent Technologies, USA). This was coupled to a 5975 inert MSD (Agilent Technologies, USA), and a MPS2 autosampler (Gerstel, Germany). The fibres were conditioned in a GC injection port of 250 °C for 30 min before use. The sequence of the analysis is shown in Figure 4, and had a total of 31 steps. Analysis was repeated three times on the same sample vials, with start times at 0, 4 and 7 DAI, and each had a total run time of 36 h and 59 min per analysis sequence.

The SPME headspace sampling was performed with a vial penetration depth of 31 mm, 40 min extraction time, and desorption for 60 s with an injection penetration by the needle of 67 mm. This included 3 min of fibre bakeout in the second inlet at 250 °C before extraction, and 15 minutes after injection, to avoid carryover. No incubation parameters in the form of agitation or heat were applied on the sample vials before extraction.

Analysis sequence:

Analysis steps:

Figure 4: Analysis sequence and number of steps in each analysis of E1. The labels are explained in more detail in Table 3. This sequence is repeated on 0, 4 and 7 DAI.

2.4.2 Experiment 2

Eight cucumber plants were moved to the opposite side of the growth chamber, to avoid contamination of healthy plants, and strung up to ropes in a similar manner as explained in Chapter 2.3.1. Some withered leaves were removed from the plants, as well as new side shoots to keep them from growing too wide. The withered leaves were likely caused by the transport of plants from a bright and humid environment with less space in between plants, to an environment with lower light conditions and humidity. A total of four replicates, consisting of two plants each, were placed on a plastic tray inside separate plastic covers in the shape of tents. These were attached to a line hanging across the room and closed up by duct tape. The sides and top parts were closed and assumed airtight, while the lower part was wrapped under

the plastic trays and closed lightly with stationary tape. This to be able to open and close for ventilating before sample collection as well as for watering of the plants.

Before closing up the plastic tents on the day of preparation, the cucumber plants were watered and a small cut made on one side of each stem by a sterile scalpel, close to the root system. This step was done to ensure the control plants would produce volatile signals as similar to the inoculated plants as possible.

Collection of volatiles was done by static sampling, and at approximately the same time each day in the morning. The first samples were taken on the second day, from the headspace of healthy control plants. The plastic cover was opened at the bottom for watering and letting the tents air out. After one hour the plastic was closed back up. Four metal stands were lined up, one beside each tent. After finding the right placement for all four 2 cm 50/30 μm DVB/CAR/PDMS fibres (Supelco by Sigma-Aldrich, Germany), in a location where they had enough space for the length of the fibres, the needles were fixed in place by lab tape on top of each tray at a height of 65 cm. The needles were allocated to specific tents, and both marked accordingly with a number from one to four. Needles were pierced to a depth of 2-3 cm into the headspace, a timer was set, and fibres extended and exposed one by one.

Figures 5A and B: Pictures showing the plastic tent configuration, each containing two cucumber plants, and a SPME needle piercing one of the tents with an extended fibre for the sampling of VOCs from the headspace.

After the extraction time of 30 min, the fibres were retracted into their respective needles in the same order, removed from the plastic tents, and immediately capped by 23Ga PTFE SPME sealing caps (Supelco by Sigma-Aldrich, Germany). Holes in the plastic tents were sealed by a small piece of tape to prevent air from escaping. The capped fibres were wrapped separately in aluminium foil and placed inside of a sealed plastic bag. This was kept on ice in a Styrofoam box as an extra measure to prevent volatiles from escaping the fibre, and to reduce variation between samples, before being transported to the instrument lab at UiS for analysis by GC-MS.

Immediately after the first sampling of healthy control plants, all replicates were infected with the fungal pathogen *P. aphanidermatum.* The reason for using the same plant replicates as for the healthy control on the previous day, was to reduce the variation between samples before and after inoculation, compared to using a second set of plants. Also, the experiment was limited to four individual SPME fibres. The inoculation was done by making a cut with a sterile scalpel on the opposite side of the stem as the cut from the previous day. Afterwards a thin piece of PDA containing the pathogen was placed directly onto both cuts that were made. The pieces of agar were of approximately 0.5 cm^2 and covered in fungal material from a nine-day old culture with good growth. After attaching the agar pieces, a black sheet of plastic was placed over the lower part of the stem of each plant to provide a dark environment for the fungi to develop. The plastic covers were closed up, and the first sampling of infected plants was performed the next day, in the same manner as explained above. Care was taken not to water on top of the agar pieces the following days. Monitoring of any visual signs of infection by *Pythium* sp. was also performed each day before watering of the plants.

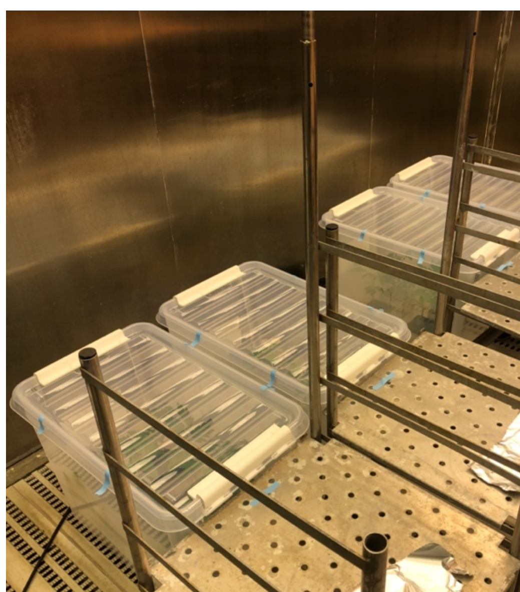
2.4.3 Experiment 3

Due to the lack of plant- and microbe emitted volatiles from the application of static sampling in E2, it was decided to use a dynamic sampling method for E3. Four 61 litre plastic boxes that measured 60 cm in length, 40 cm in width and 42 cm in height were acquired. A hole was drilled on both sides of each box, one at the bottom corner of the backside for inserting the tube from the air pump, and one at the middle of the front side for injecting of the SPME needle while sampling. One EHEIM Air Pump with an effect of 200 l/h (2x100 l/h) was connected to each of the plastic boxes to create air flow for the dynamic sampling. Plastic tubes were joined to connect the two inlets producing 100 l/h each. Four replicate plants of 28 days growth were

placed inside each box. The plants had grown to a height of approximately 20 cm, including the rockwool substrate cubes. In addition, approximately 15 extra healthy plants were kept in the same growth chamber, to observe any changes in physical development of the plants inoculated with the pathogen.

All 16 tomato plants which were placed into boxes, had two of their largest leaves pricked five times with the tip of a sterile scalpel. This was done to the healthy control plants to imitate the volatile release for infected samples the following day. Plants were watered to full capacity of the substrate. Lids were left loosely on the boxes.

The next day, all lids were taped shut and pumps were started. After 20 minutes of airing the boxes out, the 2 cm 50/30 µm DVB/CAR/PDMS fibres (Supelco by Sigma-Aldrich, Germany) were assembled on stands of the same height as the sampling hole and fixed to the trays by lab tape. Airing of the boxes was done to eliminate volatiles that had collected over the last day, so that the collected VOCs would be the ones recently emitted from the plants and thus give a better picture of the current disease development. Fibres were extended into the boxes one by one, and after 30 min of extraction, the fibres were retracted into their respective needles in the same order, removed from the boxes, and immediately capped by 23Ga PTFE SPME sealing caps (Supelco by Sigma-Aldrich, Germany). Fibres were transported to the instrumental setup in the same manner as described for E2 in Chapter 2.4.2.



Figures 6A and B: Photos showing the arrangement of the four boxes with corresponding sampling trays, fibres attached by blue lab tape, and the placement of tomato plants inside.

Plants were placed to avoid contact with other leaves and the walls of the boxes as much as possible. The inserted tube coming from the air pump, can be seen in the top left corner of figure B. This placement was chosen to force the airflow from the bottom and up towards the sampling hole.

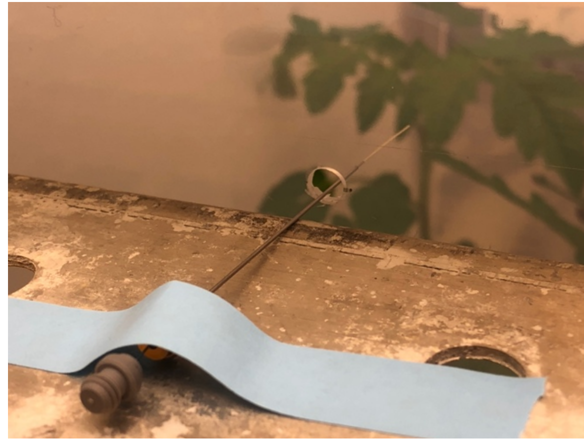
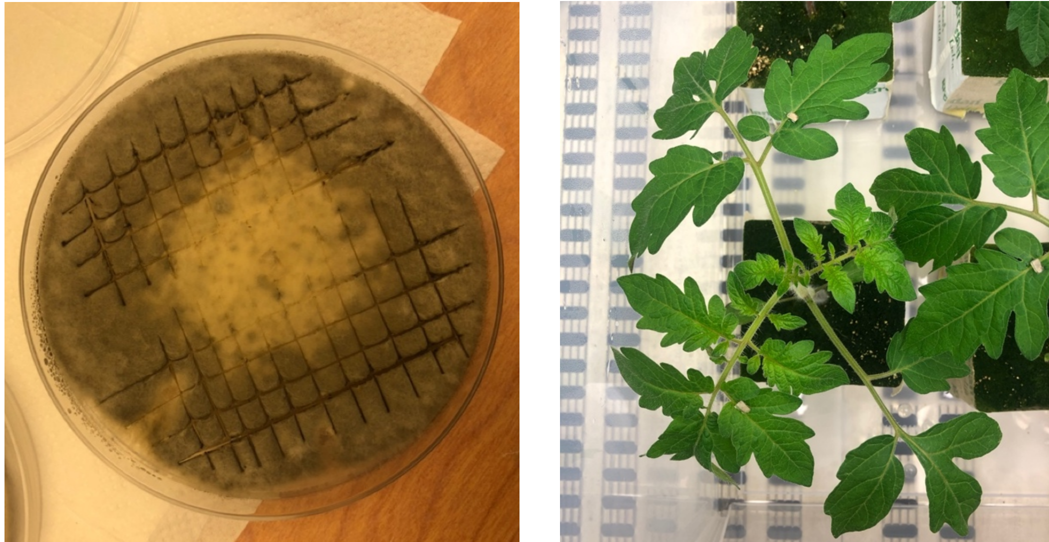


Figure 7: Sampling hole in one of the plastic boxes, with the inserted and fixed SPME needle and exposed fibre.

On the same day as the sampling of healthy controls, tomato plants were inoculated with *B. cinerea.* This procedure was started by again pricking two new leaves of each plant with the tip of a sterile scalpel. A thin piece of agar of approximately 0.5 cm^2 was places upside down on each of the newly damaged leaves. The agar pieces were cut out from an PDA plate of *B. cinerea* which had been growing for 11 days (Figures 8A). The lids were loosely placed back on the boxes, and plants left for the next day. Sampling on 1-5 DAI was performed in the same manner as described above. The temperature inside the boxes was also monitored outside of sampling times, to make sure it did not differ too much from the room temperature.



Figures 8A and B: Photos showing the PDA plate of B. cinerea with a square pattern of agar pieces to be placed on the leaves of the tomato plants. The fungi were placed on the wounded leaves, close to the main stem of the plant.

The amount of time needed for airing out the boxes prior to sampling, was calculated by dividing the volume of one box by the capacity of the pump. During aeration one full volume of the box was replaced, which took a maximum of 20 min. The inserted tube originating from the air pump was placed at the bottom corner of the box to avoid touching of the plants, as well as aiding the trajectory of airflow upwards in the direction of the sampling hole on the opposite side, moving through the plants. The hole drilled for the tube was placed a few centimetres over the base in case of some water accumulation.

On the sixth and last day of infection, an experiment was performed to find out if candidate biomarker volatiles detected inside the boxes could also be discovered in the larger volume of air of the entire growth chamber. The collection of VOCs was performed in the ventilation shaft of growth room 11 containing the tomato plants, including a control from the ventilation of room 12 which previously held the non-infected cucumber plants. Both rooms had a length of 3.98 m, width of 2.25 m and height of 2.60 m. By calculation, this gave a volume of 23.28 m^3.

The ventilation produced an air flow of approximately 9 m/s for both rooms, measured by an anemometer inside each of the ventilation shafts. At the individual sampling sites, the flow was approximately 4 m/s for room 11 and between 2-3 m/s for room 12. A contraption to hold the SPME fibres for the duration of the sampling time was made of a laboratory rack with an

attached piece of mesh, and a weight at the base. This was used to hold the SPME needles in place and horizontal during the collection of VOCs. Three needles were used for extraction in air from the room containing the diseased tomato plants, at time points 30, 60 and 90 minutes. The fourth needle was assigned as a control to extract volatiles from the air of room 12 for 90 min.

Figures 9A and B: Setup of sampling systems inside of the ventilation shafts of room 11 (left) and room 12 (right). Air flows from the hole and continues through the filter at the right of both photos. SPME fibres were fixed through a piece of mesh to keep them perpendicular to the air trajectory.

2.5 GC-MS analysis of VOCs

After optimisation of the analytical method in Chapter 3.1, some modifications were also made during the experiment. The same instrumental settings were used for all three experiments, but the exposure time of SPME fibres was adjusted depending on the sampling type. The sampling procedure for E1 is explained in Chapter 2.4.1. For E2 and E3 sampling was performed at the greenhouse location, and SPME fibres with the sample material transported to the instrument lab. Following transport of the fibres, one at a time was unwrapped and assembled into an automatic fibre holder and placed into the arm of the MPS 2 autosampler (Gerstel, Germany). An injection penetration of 67 mm was selected for the desorption of 60 s in an inlet of 250 °C. For the part of E3 where samples were taken in the ventilation shaft, the desorption was increased to 120 s due to the smaller amounts of the volatiles of interest in a growth room compared to the smaller boxes.

Analysis was performed on an Agilent Technologies 6890N GC (USA) coupled to a 5975 inert mass selective detector (MSD), with a MassHunter GC-MS acquisition software

(B.07.02.1938, Agilent Technologies, USA). Injection of samples into the GC were made in splitless mode with a glass inlet liner of 1 mm internal diameter (1 mm ID SPME Liner for Gerstel CIS4 inlet, prod. nr. 011712-010-00), and the collected volatiles were separated on a Rxi-5Sil MS capillary column of 30 m x 0.25 mm i.d. x 0.25 µm film thickness (Restek, USA). Oven temperature conditions were initially replicated from the method of Cortina et al. (2017), but due to some instrument malfunctions related to the cooling of the GC oven, the starting temperature could not reach 35 °C and was increased to 37 °C for all analyses. This also resulted in a shift in some of the ramping timepoints compared to the original method.

The resulting oven conditions were as follows; 37 °C for 5.67 min, 3 °C/min ramp until 45 °C, 1.5 °C/min ramp until 50 °C, held for 1.5 min, 3 °C/min ramp until 68 °C, held for 2 min, 3 °C/min ramp until 131 °C, held for 1 min, and finally a 10 °C/min ramp until 250 °C, held for 2.93 min, with a total run time of 58 min for each chromatographic analysis. This was followed by a post run at 37 °C for 1 min. For E1, the post run was increased to 10 min due to a sequence delay caused by the cooling time of the oven. Electron ionization mass spectra were recorded at frequency 11.1 scans/s with a scan range from 35-250 m/z, in the MS scan acquisition mode.

A mixture of alkanes from C8-C40 (Supelco by Sigma-Aldrich, Germany) was run with the same parameters as above to calculate a retention index. Alkanes from C8-C19 were identified by TIC and spectral identification using NIST MS search 2.0 (DB v. 2011). The alkanes were listed in a TXT file with corresponding retention times, in the format of Kovats retention index. This file was in turn uploaded into projects in untargeted metabolomics software MS-DIAL version 4.38 (Tsugawa et al., 2015), for comparison of the deconvoluted peaks in E1 and E3.

For the targeted analysis of candidate biomarker compounds in E3, a selected ion monitoring (SIM) scan acquisition mode was applied for the MSD, in addition to the scan mode explained above. When detecting small concentrations of compounds in a larger space such as the full volume of a growth room, a more sensitive method is required. In SIM mode the instrument scans with a higher sensitivity at certain time points for the specific masses of interest. Considering the four compounds in Table 5 were not yet identified, six SIM groups were included in the analysis. The three relevant groups later identified included the masses 93.0, 105.0, 121.0 and 136.0 m/z from 18 min (related to compound ID105), masses 77.0, 91.0, 93.0 and 136.0 from 20.5 min (related to compound ID123), and masses 105.0, 119.0, 161.0 and 204.0 from 39 min (related to compounds ID220 and ID223).

2.6 Data processing and statistical analysis

After data acquisition of volatile compounds from the three separate experiments using the MassHunter GC-MS software, all raw sample data files were converted to an appropriate file format (ABF) for uploading and data pre-treatment in MS-DIAL ver. 4.38. MS-DIAL is a universal data software for untargeted metabolomics analysis that supports data from various instruments including GC-MS. Projects were created for E1 and E3, separately. E2 was not subjected to further data processing due to the lack of relevant results. For most analysis parameters in MS-DIAL the default setting were used. The exceptions included mass scan range which was adjusted to 35-350 Da, and the fact that retention index (RI) was applied instead of retention time (RT) for identification and alignment parameters. The alkane index file previously created from the mass spectra of alkanes C8-C19 was also uploaded as a means to convert retention times into independent constants. The alignment reference file was selected based on which sample in the current project contained the highest total number of peaks.

The processed data was grouped by sample and treatment type and the alignment normalised by total ion chromatogram (TIC) in MS-DIAL. The alignment results were exported as raw data matrix (height) in deconvoluted spectra type and MSP file format. The export was then opened in Microsoft Excel for manual filtering and formatting prior to statistical analysis. The manual filtering steps in Excel was performed to discard compounds of low significance based on p value acquired from MS-DIAL and removing the background signal by subtraction of the blank, which resulted in a significant reduction in number of peaks before further processing. Peaks with a p value higher than 0.05 were regarded as less significant and thereby excluded, identified duplicates were removed based on signal-to-noise ratio, fill percentage and fold change of the peaks, and blank subtraction was performed.

The final Excel sheet was converted into comma separated values (CSV) format and uploaded to the statistical analysis software MetaboAnalyst ver. 5.0 (https://www.metaboanalyst.ca) which in this case was used as a visualisation tool. Data filtering was based on interquartile range (IQR), normalisation by sum, a generalised log transformation was applied, and pareto scaling (mean-centred and divided by the square root of standard deviation of each variable).

One-way analysis of variance (ANOVA) was performed for the data sets, followed by several steps of multivariate analysis including and a correlation heatmap with dendrogram.

Principal component analysis (PCA) is an unsupervised pattern recognition technique that shows the difference between treatments of the samples. Partial least squares discriminant analysis (PLS-DA) is a supervised method applied for stronger separation, and the identification of compounds which contribute most to the observed differences is provided by VIP scores. Orthogonal partial least squares discriminant analysis (OPLS-DA) is a variant of the PLS-DA where a regression model is constructed between the multivariate data and a response variable that only contains class information. A single component serves as a predictor for the class, while the other components describe the variation orthogonal to the first predictive component (Creative Proteomics, 2021). This can make for an even better separation by OPLS-DA compared to the other methods of analysis.

PLS analysis is followed by variable importance in projection (VIP), a strong tool for identifying which of the significant compounds contribute most to the observed differences. VIP is calculated for each of the components included in a data set and estimates the importance of each variable in the estimation of a PLS model (MetaboAnalyst, 2021).

3 Results

3.1 Development and optimisation of HS-SPME-GC-MS method

3.1.1 Literature review

The analytical procedures of collecting and analysing VOCs emitted from the fruits and plants of selected cucumber and tomato varieties, were optimised based on literature review and continued practical assessment of the applied methods during experiments. Not many articles from the literature review explaining the details of HS-SPME sampling of tomato leaves could be located. But due to the similar composition of tomato fruits and tomato leaves, articles describing the methods of obtaining volatile profiles from tomato fruits were found useful. Also experiments of other types of plants were included to show a broader range of sampling from these kinds of tissues.

Details of special importance in the literature review were tissue preparation, volatile collection method, which kind of VOCs were collected, SPME type, analytical method and computer software. These details were gathered from several sources and can be found in Table 6 (Appendix A). The mentioned table shows an overview of frequently used methods for analysis of specific VOCs. It contains collection methods and analytical methods performed for different plant species. By summary of the 11 reviewed articles, it is most common to use automatic sampling for HS-SPME. Larger vials of 10 or 20 ml are frequently used to hold the samples. Times and temperatures for pre-incubation, extraction and desorption of the samples vary quite a lot depending on the sample materials. Pre-incubation is not a relevant feature for this project, as sampling will take place either from the greenhouse site or from the same sample vials at different time intervals. Extraction times varied between sample types, along with the molecular weight of the actual compounds of interest. Desorption happened most frequently for 1-2 min and at a temperature of 250 °C. Splitless seemed to be the most used type of injection in these cases.

The GC-MS methods also show some varieties in the configurations of the different experiments. All of the articles mentioned used a capillary column of 30 m x 0.25 mm ID x 0.25 µm film thickness or 60 m x 0.25 mm ID x 1.0 µm film thickness. The carrier gas was

most commonly helium with a flow rate of 1.0 or 1.2 ml/min, and the ionisation energy was also a constant at 70 eV. Mass acquisition ranges differ between the experiments but keep somewhat in the same area, while for the oven temperature settings there is a large diversity. The most used fibre mentioned in the articles from Table 6 is the 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre type. This seems to provide a greater mass acquisition range than other similar ones and gives a greater sensitivity for the highly volatile compounds such as terpenes.

3.1.2 Sampling and injection techniques

The sensitivity of sampling and injection techniques are important for the analytical procedure, due to low concentrations of specific VOCs which can occur in a greenhouse setting with monitoring of plant-related disease. Headspace (HS) coupled to solid phase microextraction (SPME) sampling is a generally easy and fast tool which provides a clean chromatography result (Tipler, 2020). SPME fibres should be chosen to match the MW of the compounds of interest to be detected. Fibres of 1 cm length is the most common, but in this case a 2 cm DVB/CAR/PDMS fibre was chosen for even better sensitivity for VOCs.

3.1.3 Analysis by GC-MS

The GC-MS analysis parameters were based on the method of Cortina et al. (2017). The reason for this choice was mainly based on the similarity of instrumental setup, and especially a GC column of the same size. The resulting analysis parameters which were applied for experiments of this project, can be found in Chapter 2.5.

3.2 Cultivation of fungal pathogens

The first transfer of *Pythium* spp. cultures to new PDA plates was executed by streak plate technique, using a metal rod, gas burner and 70 % EtOH. Agar plates were wrapped with Parafilm to avoid contamination, and incubated upside down at 25-30 °C. This method produced no new growth on the media, and therefore another method was tested. For the next attempt of subculturing (explained in detail in Chapter 2.3.2), a sterile scalpel was used to cut small pieces out from the agar containing fungi, which was placed in the middle of new PDA

plates with the mycelial side down. All other parameters were kept constant. This produced good fungal growth and was used forward for subculturing.

From the two tests of pathogenicity (see Chapter 2.3.2), *P. aphanidermatum* was chosen for further experiments on cucumber fruits and plants, and *B. cinerea* isolate BC 96/19-2.8 for all experiments related to tomato fruits and plants. These specific isolates were chosen based on characteristics such as being fast growing and producing large amounts of fungal material in fruit tissue or on agar plates (more details in Table 2, page 22).

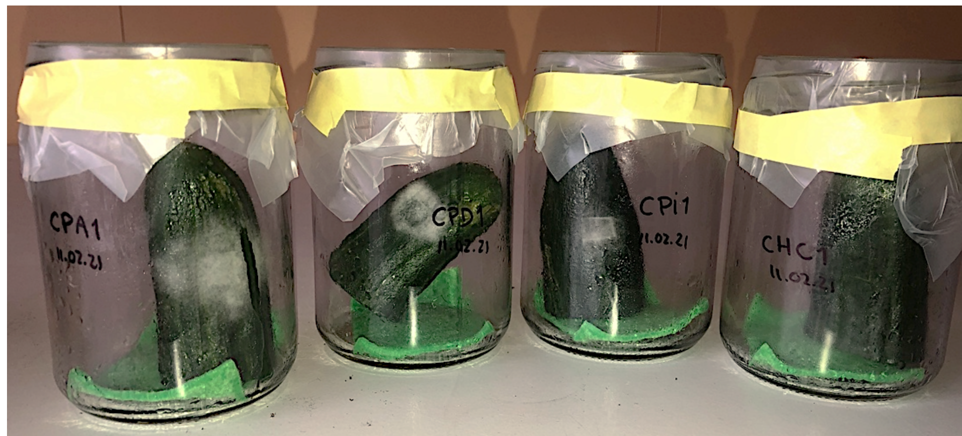
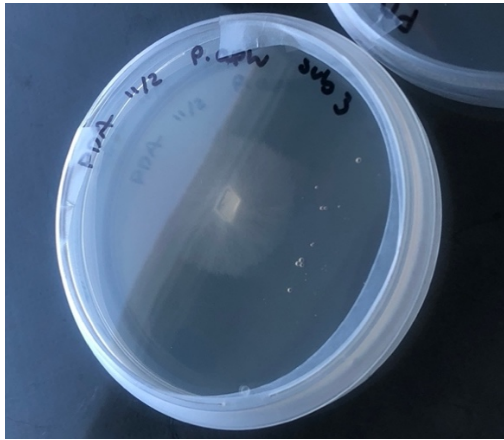
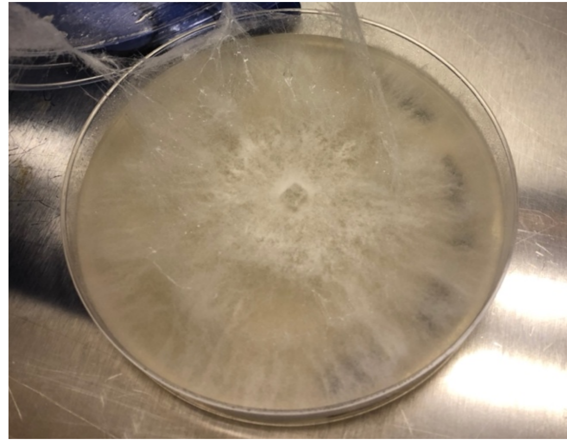


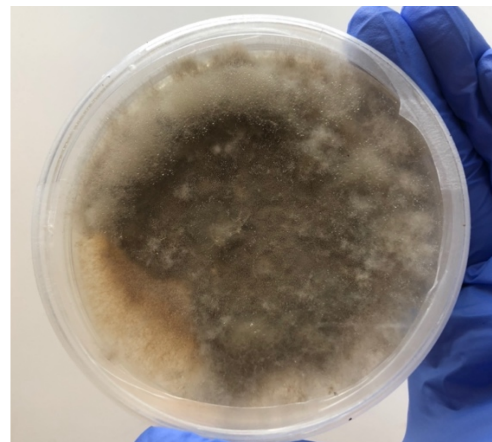
Figure 10: Test of pathogenicity on pieces of cucumber, 5 DAI. From the left the jars are labelled CPA (inoculation with P. aphanidermatum), CPD (inoculation with P. dissotocum/coloratum), CPI (inoculation with P. irregulare), and CHC (healthy control).

On PDA growth medium, the observed morphological characteristics of *P. aphanidermatum* were filamentous, thread-like structures with lots of mycelial strands. It showed a pattern of growing radially from the centre of the agar plate, and a colour close to white. The growth of *B. cinerea* on the same type of medium was slightly less filamentous, with a darker colour of grey which grew darker as time progressed. This fungus appeared in spots on the agar, before developing into the fuzzy structure shown in Figures 12A. Both *P. aphanidermatum* and *B. cinerea* seemed to produce vegetative mycelium, which grew into the medium to absorb nutrients, and aerial mycelium extending over the surface to form conidia for reproductive purposes.





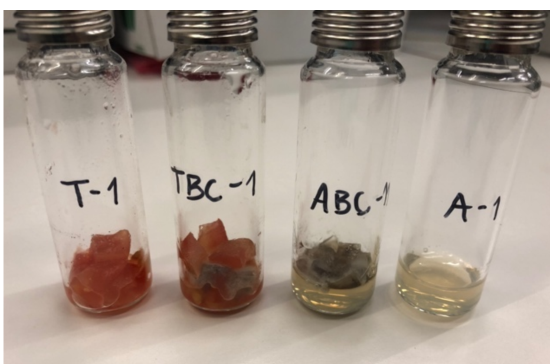
Figures 11A and B: P. aphanidermatum fungal cultures, one and three days after subculturing and incubation at 25-30 °C. The cultures are from passage number three and two, respectively.

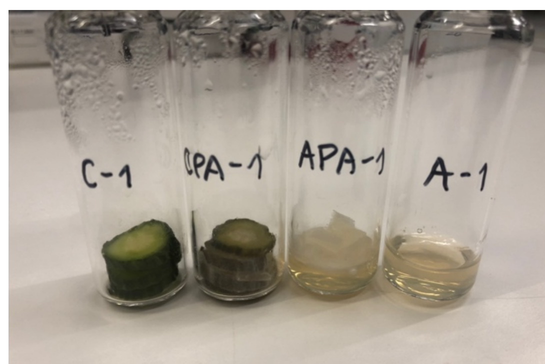


Figures 12A and B: B. cinerea fungal cultures at seven and nine days after subculturing and incubation at 25-30 °C. The cultures are from the first and second passage, respectively.

3.3 Analysis of VOCs emitted by pathogen infected cucumber and tomato fruits in laboratory conditions

From visual observations of the vials prepared and analysed in this experiment (E1), as well as the data processing explained in the chapter, it was concluded that an infection was present in tomato fruit by *B. cinerea,* and in cucumber fruit by *P. aphanidermatum (*see Figures 13A and B). Because of this result, as well as the two tests of pathogenicity, it was decided to use the same two fungal pathogens to infect plants in following experiments. Cucumber plants were inoculated with *P. aphanidermatum* in E2 (Chapter 3.4), and tomato plants with *B. cinerea* (Chapter 3.5) to look for possible disease biomarkers.





Figures 13A and B: Disease progression 8 DAI for tomato fruit inoculated with pieces of PDA containing B. cinerea and for cucumber fruit inoculated with pieces of PDA containing P. aphanidermatum. Both tissues show visible signs of fungal disease, as both the tomato and cucumber fruit pieces have become depleted of moisture and also gone through a colour change. The change in colour is more easily visible in the cucumber tissue that has turned brown, while the infected tomato also has taken a slightly darker tone.

The average number of peaks for each sample type after deconvolution by MS-DIAL is listed in Table 4 below. The general trend is an increase in the number of peaks starting from the first to last day of analysis. The number of peaks is also higher for samples that contained pieces of fruit as opposed to samples containing PDA instead. The full list of 104 peaks with their corresponding retention times, retention indexes and quant mass obtained from the alignment results in MS-DIAL, is included in Appendix B, Table 7. This list shows the deconvoluted peaks from the part of the experiment which involves tomato and *B. cinerea*. The corresponding list for cucumber and *P. aphanidermatum* samples is not considered relevant due to the following results of E2 (Chapter 3.4) and thus not included.

Table 4: Average number of peaks after deconvolution for the samples ABC (B. cinerea on agar), TBC (B. cinerea infected tomato fruit), APA (P. aphanidermatum on agar) and CPA (P. aphanidermatum infected cucumber fruit).

Day	**ABC**	**TBC**	**APA**	**CPA**
1	51	93	27	77
5	64	81	42	114
8	67	115	45	185

After data filtering in MetaboAnalyst, the 104 peaks detected after *B. cinerea* infection in tomato fruit was reduced to 75 peaks (see Figure 14). This decrease was the result of blank subtraction prior to the metabolomic data analysis, and the subsequent filtering out of missing values.

	Features (positive)	Missing/Zero	Features (processed)
DAY1-ABC-1	31	45	75
DAY1-ABC-2	32	44	75
DAY1-ABC-3	20	56	75
DAY1-ABC-4	32	44	75
DAY1-TBC-1	20	56	75
DAY1-TBC-2	27	49	75
DAY1-TBC-3	21	55	75
DAY1-TBC-4	19	57	75
DAY5-ABC-1	22	54	75
DAY5-ABC-2	24	52	75
DAY5-ABC-3	23	53	75
DAY5-ABC-4	21	55	75
DAY5-TBC-1	22	54	75
DAY5-TBC-2	39	37	75
DAY5-TBC-3	28	48	75
DAY5-TBC-4	30	46	75
DAY8-ABC-1	58	18	75
DAY8-ABC-2	63	13	75
DAY8-ABC-3	55	21	75
DAY8-ABC-4	56	20	75
DAY8-TBC-1	61	15	75
DAY8-TBC-2	65	11	75
DAY8-TBC-3	61	15	75
DAY8-TBC-4	64	12	75

Figure 14: Summary of data processing results after data filtering in metabolomic data analysis with MetaboAnalyst 5.0. An Excel file containing a data matrix of 24 samples by 104 peaks (mz/rt) from the E1 alignment results in MS-DIAL was uploaded and processed. Peaks are labelled as features in this table.

3.4 Untargeted analysis of VOCs released from *Pyhtium* sp. infected cucumber plants using static sampling

The cucumber plants did not show clear signs of *Pythium* infection throughout the second experiment. An experienced adviser in greenhouse production suggested that infection had not occurred due to the fact that it should be clearly noticeable if present on the stem of the plant.

It was also determined that the acquired sets of chromatograms generally showed a very low number of peaks due to a lack of sensitivity in the experimental method and low concentration of VOCs. In addition to the low number of detected peaks, most of these were assumably produced by injection of atmospheric gases and column bleed products. Most of the peaks present in the chromatograms for E2 samples are also present in samples from other experiments. Figures 16A and B illustrate the relationship between E1 and E2 in this regard.

As the majority of detected peaks originated from inorganic sources and mainly column bleed products, the exploration of identifying peaks was not taken further. As illustrated in Figure 15, the difference between the first and last day of sampling is not progressing noticeably along with the experiment. The low number of detected plant related volatiles was likely a result of

the static sampling procedure applied in the E2. For this reason, the following experiment on tomato plants was executed by dynamic sampling.

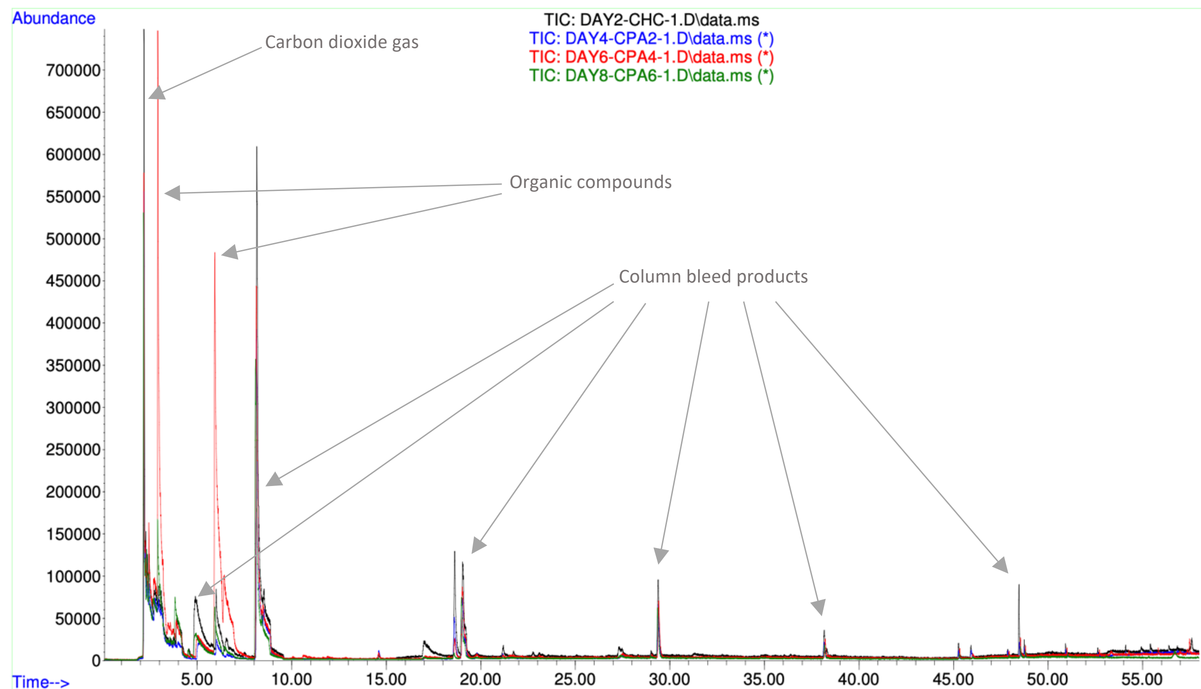
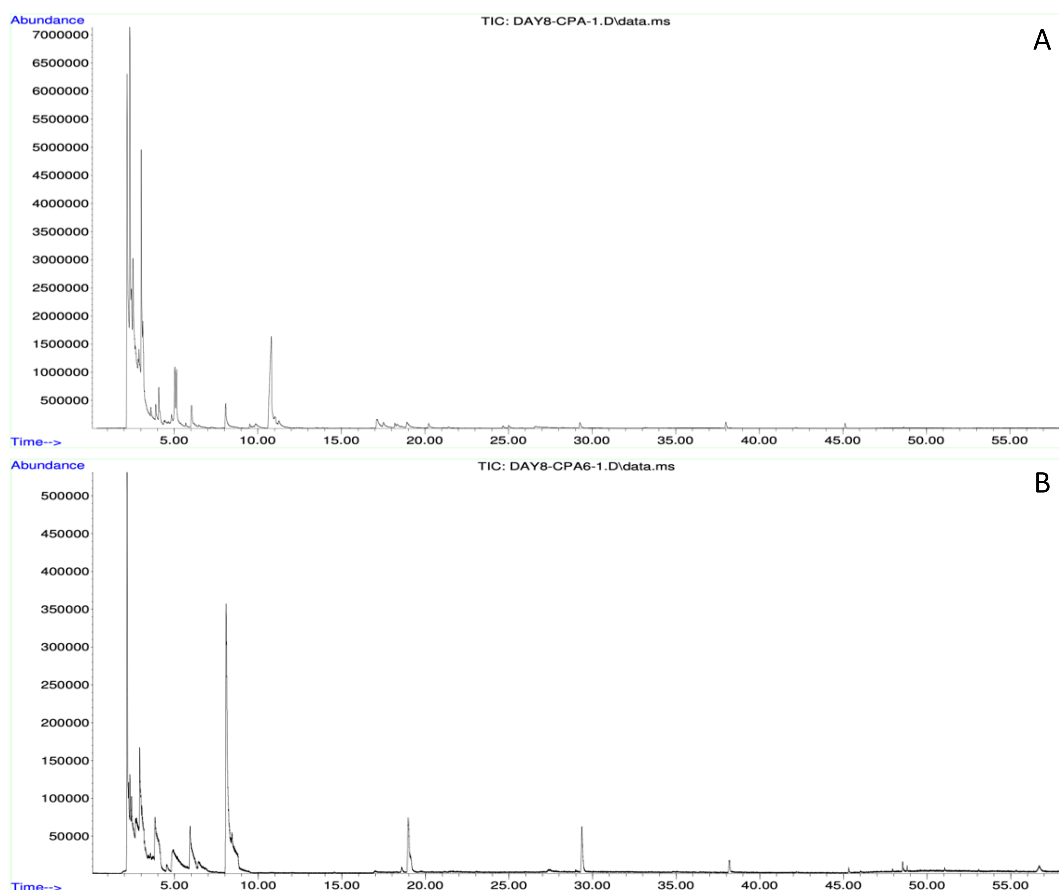


Figure 15: Overlay TIC chromatogram showing the volatile profile of sample number 1 for the healthy control (day 2) and 2, 4 and 6 DAI (days 4, 6 and 8) of the second experiment. This chromatographic overview is chosen as a representative dataset for all samples of E2, as they generally showed similar trends. The majority of peaks were identified as column bleed products and other inorganic sources.

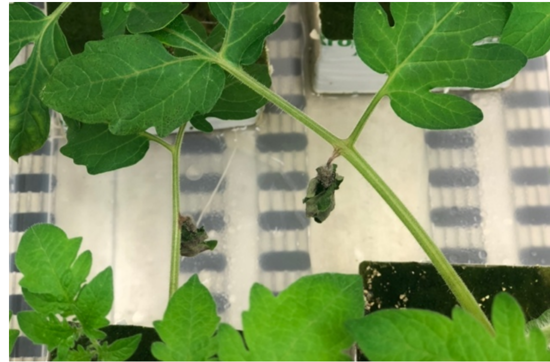


Figures 16A and B: TIC for a sample from E1 (DAY8-CPA-1) and a sample from E2 (DAY8-CPA6-1), respectively. This shows a comparison of the chromatograms which both include a number of atmospheric gases and column bleed products, but at a higher abundance in the sample from E1. Note the difference in abundance scales for the two chromatograms, which is 14 times higher for figure A.

3.5 Untargeted analysis of VOCs released from *B. cinerea* infected tomato plants using dynamic sampling

As soon as the first day after infection, the inoculated tomato leaves showed signs of disease progression. *B. cinerea* fungi were starting to grow out of the PDA pieces and onto the leaves of the plant. After the second day of infection there were significant visual signs of a pathogen attack on most of the inoculated leaves as some were caused to brown in colour and to droop (Figures 17A and B). By the fifth day after inoculation (DAI) all the infected leaves were brown, curled and wilted. The infection was localised only on inoculated leaves. After the experiment, infected plants showed some stunted growth compared to the healthy controls, and the colour of the leaves was also lighter.

Figures 17A and B: Fungal pathogen B. cinerea on tomato plant leaves 2 DAI.



Figures 18A and B: Fungal pathogen B. cinerea on tomato plant leaves 5 DAI.

Separation and detection of compounds by GC-MS produced a large number of peaks in the chromatograms for all analysed samples. An example of this is shown in Figure 19 below. The acquired mass spectra for each sample were processed and deconvoluted in MS-DIAL, and the resulting 259 individual peaks are illustrated in the alignment spot viewer (see Figure 20). The average number of peaks for each sample type is listed in Figure 21. From the mentioned figures it might seem like the healthy control plants released the highest number of volatiles the first day of analysis (day two of the experiment), and from then went through a gradual decrease as the days passed and infection progressed.

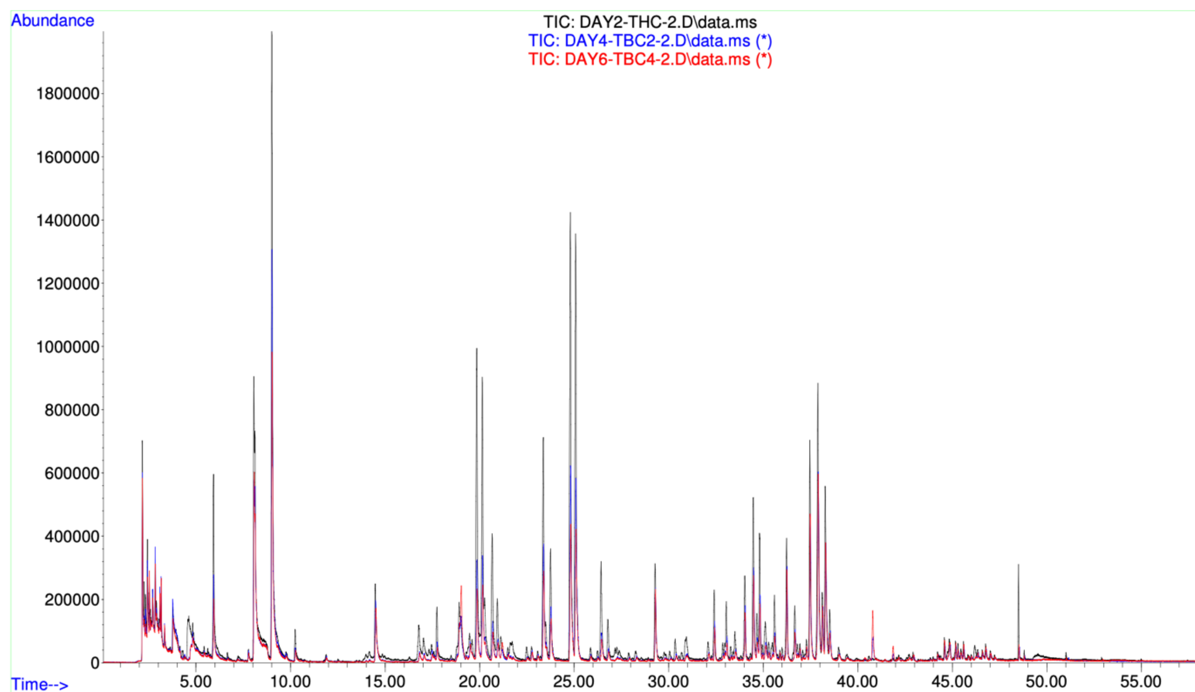


Figure 19: Representation of all acquired peaks from the overlay of three samples in E3, at analysis day 2, 4 and 6. These samples were obtained by use of the same SPME fibre.

Figure 20: Alignment spot viewer from MS-DIAL before filtering and blank subtraction, displaying 259 dots which each represent an aligned peak, including retention time, quant mass and intensity. Every peak has a unique ID number, and the colours represent relative difference in peak height.

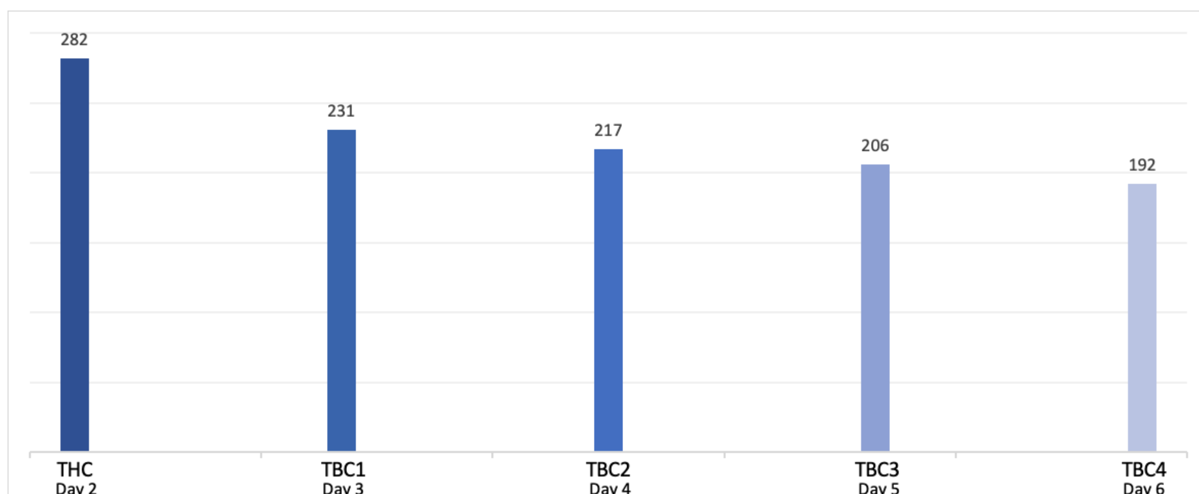


Figure 21: Diagram showing the average number of peaks from E3, after deconvolution for the samples THC (healthy control), TBC1, TBC2, TBC3 and TBC4 (day 1-4 of infection).

The 259 peaks obtained from data processing by MS-DIAL was exported to an Excel-workbook and thereby subjected to several steps of manual data filtering and blank subtraction (see Chapter 2.6 for more detailed information). The resulting data file was uploaded for further statistical analysis in MetaboAnalyst, which contained a data matrix of 15 samples by 94 peaks (mz/rt). Missing values due to blank subtraction were removed by data filtering, and the remaining 30 features were processed and normalised.

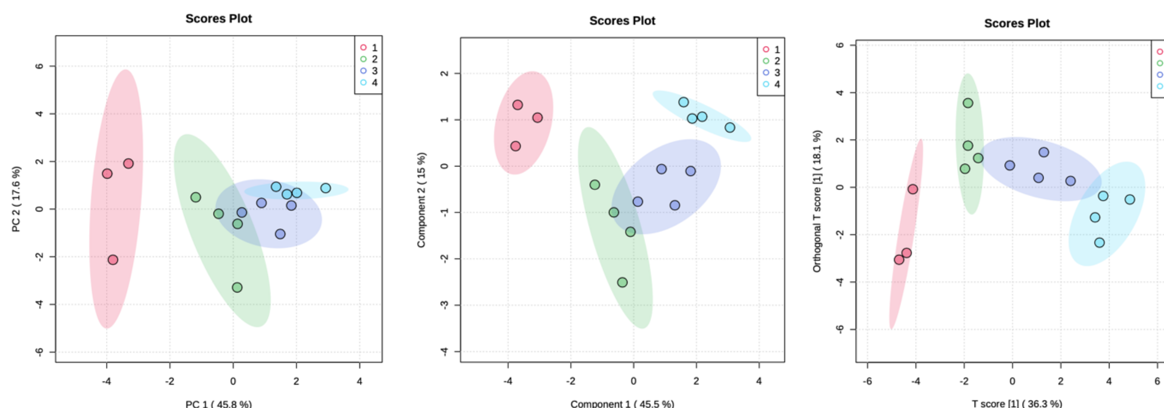
One-way Analysis of Variance (ANOVA) is a univariate analysis method and simple formula-based calculation, which determines statistical differences between several independent groups. This provides a preliminary overview of the features, or metabolites, which relative abundance were significantly different between samples. This method identified nine important features from the data set which are potentially significant. One of them, compound ID39, has a base peak of 73 m/z. This ion may correspond to the compound dimethylpolysiloxane, whose source might presumably originate from a septum or methyl silicone column bleed (Agilent Technologies, 2021). Thus, this compound is not of interest as a biomarker candidate in the experiment.

	Peaks(mz/rt)	f.value	p.value	-log10(p)	FDR	Fisher's LSD
1	39	58.61	0.00	6.33	0.00	2 - 1; 3 - 1; 2 - 4; 3 - 4
2	105	43.50	0.00	5.67	0.00	2 - 1; 3 - 1; 4 - 1; 3 - 2; 4 - 2
3	123	28.44	0.00	4.75	0.00	3 - 1; 4 - 1; 3 - 2; 4 - 2
4	223	8.92	0.00	2.56	0.02	3 - 1; 4 - 1; 4 - 2
5	178	7.60	0.01	2.30	0.03	1 - 2; 1 - 3; 1 - 4
6	149	7.19	0.01	2.21	0.03	1 - 2; 1 - 3; 1 - 4
7	220	7.13	0.01	2.20	0.03	2 - 1; 3 - 1; 4 - 1
8	162	5.60	0.01	1.85	0.05	1 - 2; 1 - 3; 1 - 4
9	106	5.55	0.01	1.84	0.05	3 - 1; 4 - 1; 4 - 2

Figure 22: List of the most important features identified from E3 by one-way ANOVA analysis.

3.5.1 Multivariate analysis

By the use of multivariate analysis, more complex sets of data which contain more than one variable can be analysed. While a univariate analysis will extract relevant features, multivariate analysis will find a group of features to make a good classification (Fan, 2018). PCA, PLS-DA and OPLS-DA analysis was applied on the results from E3 by the use of MetaboAnalyst, and the resulting scores plots are illustrated in Figures 23A, B and C below. The three mentioned analyses provided an increasing degree of separation for the four sample groups.



Figures 23A, B and C: PCA, PLS-DA and OPLS-DA scores scatter plots showing the distribution of the four sample groups with increasing quality of separation. Groups 1-4 represent 1-4 DAI. PCA and PLS-DA plots show the selected principal components with the percentage contribution for each PC in the brackets. OPLS-DA plot represent the score plot for all metabolite features. The horizontal direction of the score plots captures the variation between groups and the vertical illustrates the variation within groups.

The most important compounds in the data set determined by PLS-DA analysis are illustrated in Figure 24. 10 of the 15 compounds were excluded as a possible biomarker due to their decrease in concentration from one to four DAI. Only the compounds going from a low to high concentration were further examined. Compound ID106 was also excluded due to being identified as a duplicate of ID105.

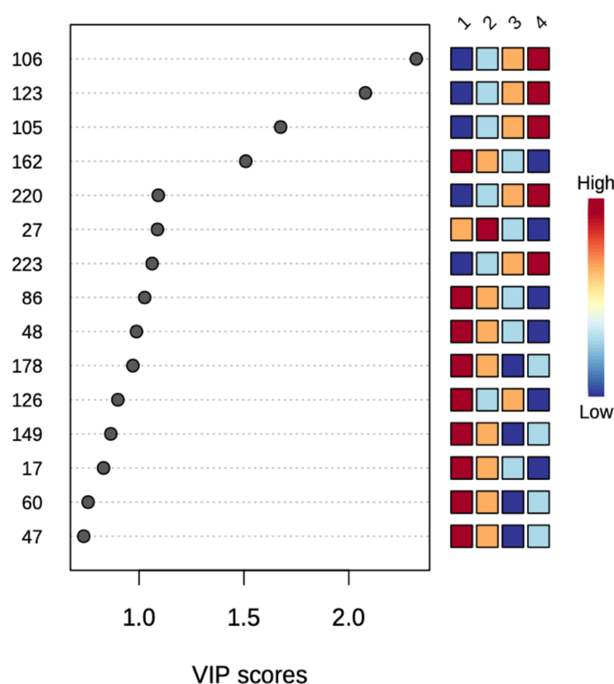
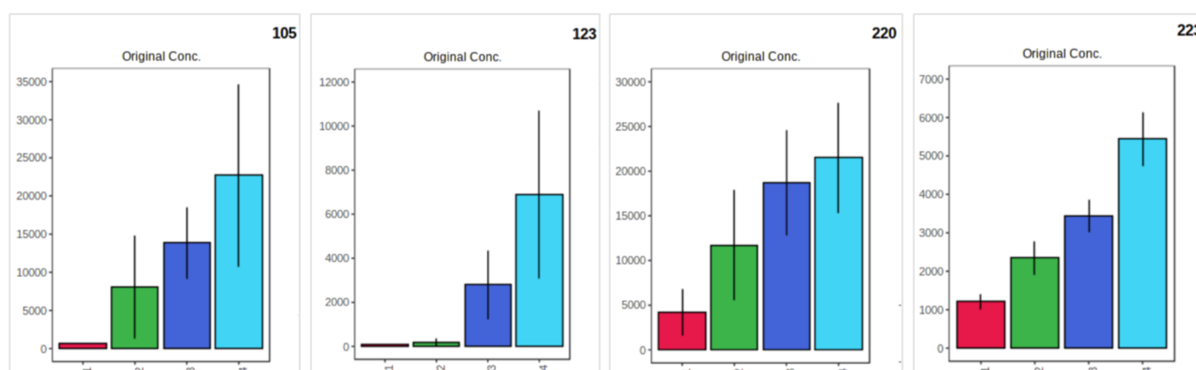


Figure 24: Important features identified by PLS-DA. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group.

The remaining four compounds were ID123, ID105, ID220 and ID223. All of these were identified by VIP analysis as gradually increasing throughout the experiment. This trend might be indicative of the release of VOCs by pathogenic infection on the tomato plants.

The four biomarker candidate compounds, as per VIP analysis, are individually visualised by box plot in Figures 25A, B, C and D below.



Figures 25A, B, C and D: The original concentrations of compounds ID105, ID123, ID220 and ID223 are shown by bar chart. All four compounds have a trend going from low to high, which is compatible with the idea of an increasing concentration of VOCs due to the plant's response to pathogenic infection.

Hierarchical clustering analysis is a method of grouping similar objects into sets of clusters. Heatmap shows the concentration of compounds by different treatments, by colour. A dendrogram is combined with a heatmap to show hierarchical relationships between the clusters. The dendrogram separates compounds into three distinctive groups where one of them contain the four compounds of interest resulting from the VIP scores (Figure 24). Both are representative for the progressing infection happening in the tomato leaves.

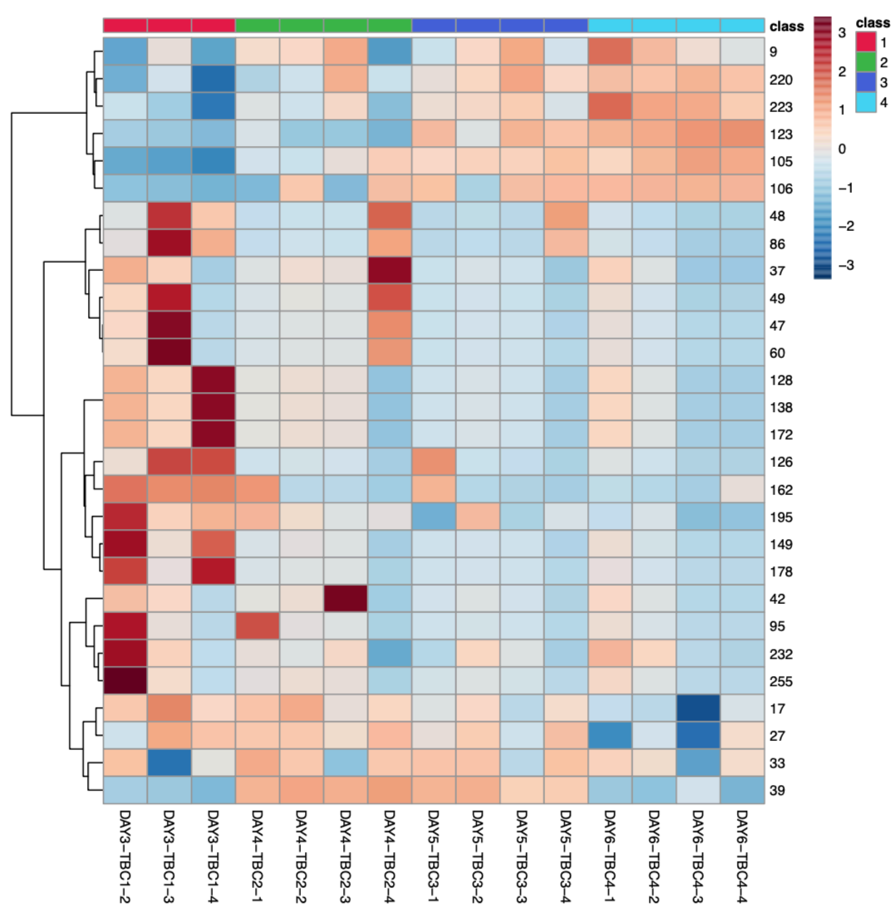


Figure 26: Hierarchical clustering results of the remaining features after data filtering in MetaboAnalyst, shown in the form of a heatmap with dendrogram to separate the groups. Sample DAY3-TBC1-1 is missing from the data set due to instrumental error of the GC-MS which destroyed the sample material during analysis. Features 9-106 increase in intensity, while 48-255 show a decrease in intensity, and the remaining four features something in between.

3.5.2 Identification of candidate compounds

After exclusion of features with decreasing concentration in VIP analysis, the remaining four compounds with the highest scores were found in individual peak list results for a representative sample in MS-DIAL. This peak list was exported in MSP file format with centroid spectra, and compound identification was performed in NIST Mass Spectral Search Program (ver. 2.0). The identification and structure were obtained by comparison of mass spectrum of the reference compound, in the NIST/EPA/NIH Mass Spectral Database (NIST 11).

Table 5: Comparison table of remaining biomarker candidates. Compound identification by NIST. MW: molecular weight, m/z: mass-to-charge ratio, BP: base peak.

Compound	Alignment ID	RT (min)	RI	Ontology	Molecular formula	MW (Da)	BP (m/z)
(+)-4-carene	105	19.04	992.5	Monoterpenes	$C_{10}H_{16}$	136	93
β-phellandrene	123	21.17	1023.0	Monoterpenes	$C_{10}H_{16}$	136	93
α-copaene	220	40.80	1369.1	Sesquiterpenes	$C_{15}H_{24}$	204	161
cis-thujopsene	223	41.88	1392.5	Sesquiterpenes	$C_{15}H_{24}$	204	119

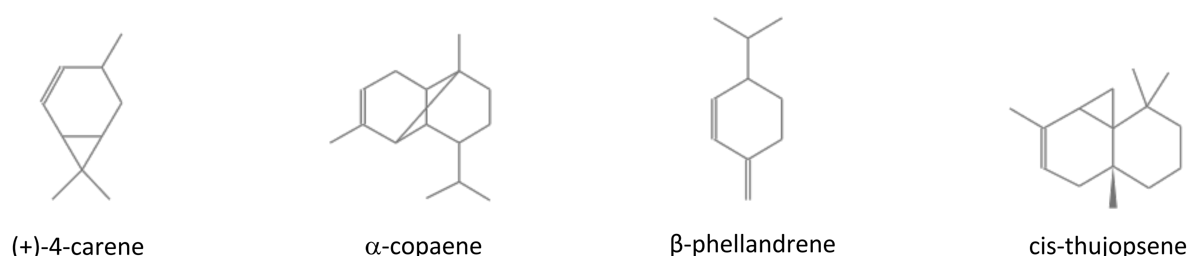


Figure 27: Chemical structures of the four biomarker candidate compounds (NIST, 2021).

3.6 Targeted analysis of possible disease biomarkers from tomato plants infected with *B. cinerea*

After identifying the retention times of some interesting candidate compounds from E3, a targeted approach was applied to look for the same compounds in a larger volume of air. The same sampling technique was transferred to the ventilation shaft of the room containing the tomato plants 6 DAI by *B. cinerea.* By including the SIM scan in acquisition of volatile characteristics, the four biomarker candidates identified in Table 5, were found to be present but in significantly smaller amounts. A small shift in RT was observed, due to the doubling of

desorption time from 60 to 120 s, but the peaks of (+)-4-carene, α-copaene, β-phellandrene and cis-thujopsene were all present with diluted peaks in the chromatograms (see Figure 28 andFigure 29 below). The observed shift in RT was 0.15 min for (+)-4-carene, 0.20 min for β-phellandrene and α-copaene, and 0.45 min for cis-thujopsene. For all four peaks, a trend of higher concentration can be seen as the exposure time increases.

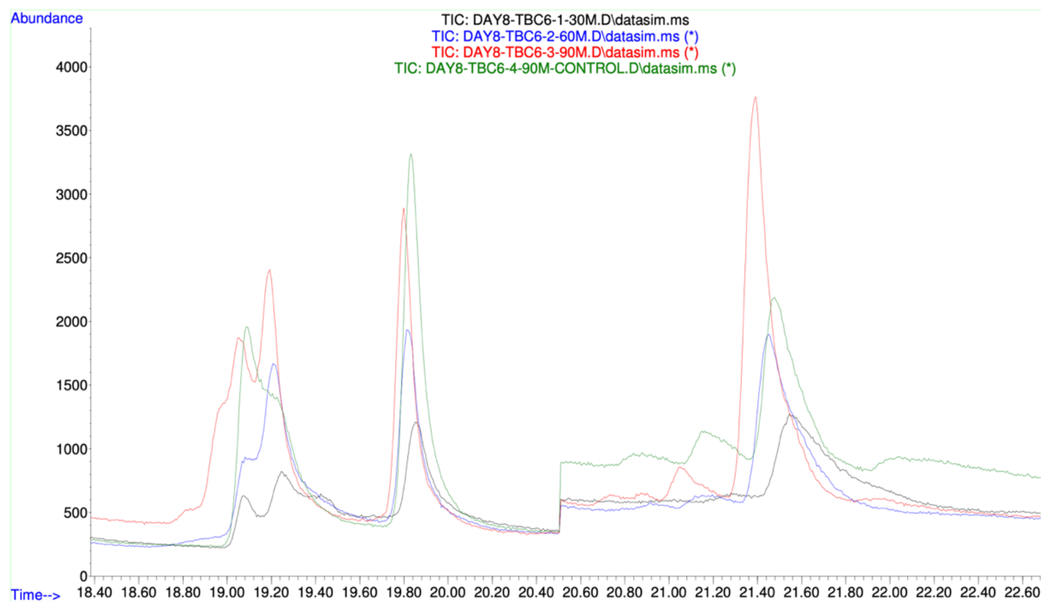


Figure 28: Chromatogram overlay of the SIM analysis at time points 30, 60 and 90 min, including a control at 90 min from a different growth room. The figure shows increasing high peaks for (+)-4-carene at RT 19.20 min and β-phellandrene at RT 21.40 min.

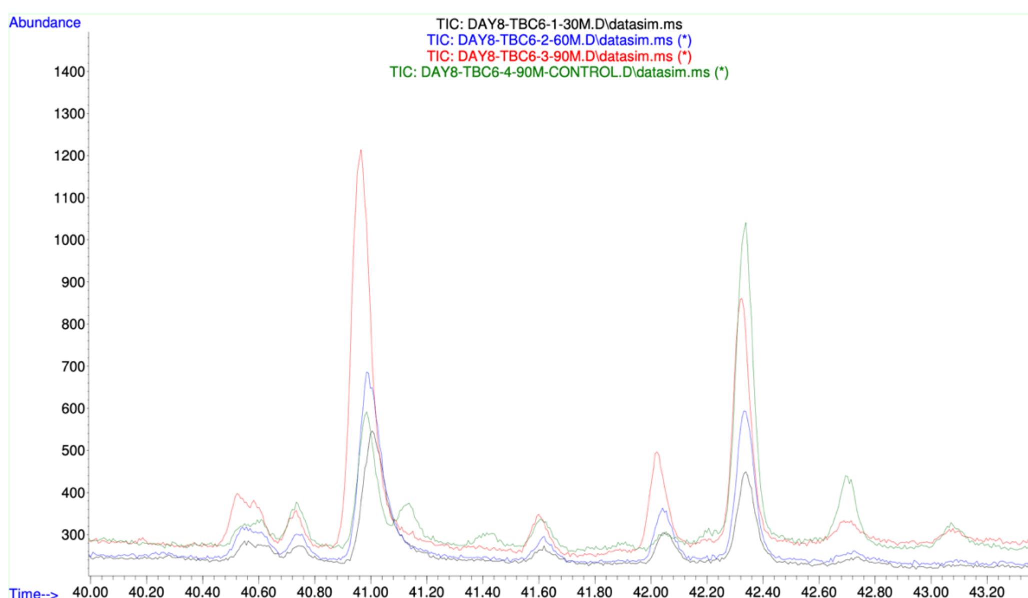


Figure 29: Chromatogram overlay of the SIM analysis at time points 30, 60 and 90 min, including a control at 90 min from a different growth room. The figure shows increasing high peaks for α-copaene at RT 41.00 min and cis-thujopsene at RT 42.30 min.

4 Discussion

4.1 Establishment of analysis for VOCs emitted by pathogen infected fruits in laboratory conditions

E1 was mainly performed as a preliminary examination of several pathogenic materials to find out which ones to use in further experiments. The two plant experiments (E2 and E3) were estimated to be more time consuming to plan and execute, and therefore it was useful to know which of the acquired fungal pathogen was most infectious for cucumber and tomato plants. Three different species of *Pythium* and four isolates of *Botrytis cinerea* were initially acquired in the interest to test pathogens known to cause serious disease for cucumber and tomato plants in greenhouse conditions, and especially associated with Norwegian agriculture. Although the isolate *B. cinerea sensu strictu* 96/19-2.8 was originally cultivated from strawberries, it has a well-known relation to the disease grey mould in tomato, and also showed good growth in tomato fruit tissues (see Chapter 3.3 for more details).

The total run time for the first analysis of experiment 1, was initially 32 hours and 20 minutes, but this was prolonged due to some delays caused by cooling down of the instrument's GC oven. The delay was approximately eight minutes per sample, so for the following analysis post run time was increased from one to ten minutes. This resulted in a total run time of 36 h and 59 min per analysis sequence. The post bakeout time was also increased from 10 to 15 min during the experiment due to some observed carryover.

The total number of peaks for E1 was increasing throughout the experiment (see Table 4), likely due to the increased emission of VOCs associated with disease development in vials containing the two pathogens *P. aphanidermatum* and *B. cinerea.* A gradual increase in number of peaks was also observed in the vials with only pathogens growing on PDA. Although this number was lower than for the vials of infected fruit, which correlates with the fact that tomato and cucumber fruits will release more VOCs than the agar used to maintain the fungi. After proceeding with E3 on tomato plants infected with *B. cinerea* it was discovered that volatile profiles did not match the profiles obtained in E1 for tomato fruits in vials, based on RT. This may indicate that *B. cinerea* infection in tomato leaves produces plant-pathogen specific VOCs, and is discussed in more detail in Chapter 4.3.

A similar approach of identifying the candidate biomarkers in E3 could also have been performed for fruit samples in E1 to find specific biomarkers related to the pathogen infection in fruits. This study was focused on looking for plant-pathogen specific compounds, so further data processing of the volatiles from fruit experiments was not pursued.

If volatiles related to the interaction between fruit and fungal pathogens were of interest, for instance during postharvest storage of fruits, it could have been useful with more extensive testing of the fruit materials or testing in greater spaces that contain larger amounts of sample materials. This is of particular interest for cucumber fruits, as these are more likely to be infected by a pathogen. The fungi might infect whole fruits in a different manner than for processed fruit pieces used in E1, which made the route of infection very available for the pathogen. Also, if fruits are damaged during transport, risk of disease development increases, which is highly relevant for the import of various kinds of fruits and vegetables. More risk factors include higher temperatures and more humid environments than recommended for storage conditions, which provides a good growth environment for pathogens such as fungi.

4.2 Identification of VOCs released from cucumber plants infected with *P. aphanidermatum* using static sampling

The resulting chromatograms from the untargeted analysis of *Pythium* sp. infected cucumber plants, subjected to static sampling in plastic tents, showed a very low number of detected VOCs. There can be several reasons for this. The cucumber plants did not show any clear signs of infection throughout the experiment, and it was concluded that infection did not likely occur at all. If an infection had been present in the plants, the total number of volatiles could potentially be higher. Low sensitivity could also be a possible explanation for the low number of detected VOCs.

The instrument showed good sensitivity for the first performed experiment, which should also have been sufficient for the analysis of plant emitted VOCs by the cucumber plants. Most peaks in the resulting chromatograms from E2 were identified as atmospheric gases, column bleed products and other inorganic sources. A possible cause for low method sensitivity was the static sampling strategy, and for this reason it was decided to use dynamic sampling in the following

experiment on tomato leaves. This should provide the SPME fibres with contact to a larger air volume for adsorption of volatiles to the retentive coating.

Due to a limited time frame, it was not possible to redo the experiment on cucumber with dynamic sampling, which could have improved detection significantly. One other thing that may have caused improvement for the initial static sampling procedure, would be to move the sampling device in a placement higher in the headspace. Collection of VOCs was performed by SPME fibres at 65 cm height. The reason for this placement was the availability of applicable fibre holders. The acquired stands had a tray height of maximum 65 cm, which placed the fibres approximately in the middle of each plastic tent. A better sampling setup where VOCs were collected at the top, might have improved the detection due to a higher temperature from lights at the top of the canopy. But because there was no ongoing infection, it would still not have been possible to detect any volatiles related to the plant-pathogen response between cucumber and *P. aphanidermatum* in this specific experiment.

Longer sampling time could also have improved the results to some degree, but it is possible that the VOCs were emitted in other locations than where sampling was performed or escaped the tents. Anyway, a dynamic sampling setup is likely a better choice, as this provides a movement of air and a better possibility of collecting VOCs into the fibre as air is passed through this space. Active sampling also provides a better picture of the current disease situation, compared to passive sampling, as accumulation of older VOCs will be purged before collection of newly released headspace volatiles inside of the plastic boxes.

It is difficult to find the reason why no infection was present on the cucumber stems. *Pythium* sp. is known for being a highly infectious fungi which spreads easily in greenhouses if the pathogen is present. It was strived to provide an optimal environment for fungal growth, with continuous watering, a humid atmosphere and to create dark conditions with no light on the lower part of the plant. It is possible that the plants were strong enough to prevent disease development, or that pieces of agar were placed too high on the stems, as the pathogen is known to cause damage in the root system. The reason for this placement was to avoid washing the agar pieces away while watering of the plant. A better alternative could have been to introduce the pathogen into the soil, where it is closer in reach of the root system of the plant, or to use less developed and thereby weaker plants or seedlings.

4.3 Identification of VOCs released from tomato plants infected with *B. cinerea* using dynamic sampling

In E3, four boxes containing four replicate tomato plants in each, were used for the dynamic sampling of VOCs. As the healthy tomato plants grew taller and wider, with larger leaves of dark green colour, the plants inside sampling boxes were somewhat stunted in growth and also of a lighter colour. This might be a result of the ongoing infection, and also the smaller space and lower light conditions inside the box. Healthy controls were placed on top of a table, and thereby closer to the lights than infected plants. Another reason for reduced growth in diseased plants, is a redirection of photosynthetic products to boost their immune system and fight the pathogenic attack. The wilting of infected leaves can also result in less overall photosynthetic capacity of the tomato plants. Infection was located only to the inoculated leaves of each tomato plant. This might differ from a less controlled environment where contamination of surrounding plants is more likely to happen. In E3, the plants were spread out inside the boxes with some space in between, and care was taken not to spread infection for instance when watering the plants.

The number of detected peaks and their intensities were significantly higher for dynamic sampling of the infected tomato plants, compared to E2. The reason for that was the collection method for volatiles, and successful disease development in the plants. The data processing steps resulted in the identification of four biomarker candidate compounds, (+)-4-carene, β-phellandrene, α-copaene and cis-thujopsene (Table 5). The two first are monoterpenes, and the two others are sesquiterpenes. These groups of compounds are related to the smell of various plants and mentioned by Thelen et al. (2005) as the largest groups related to volatiles collected after fungal infection in tomato leaves. Their research suggests the VOC α-copaene as a significantly distinctive VOC for fungal disease in tomato leaves, possibly to the specific interaction with *B. cinerea* infection (Thelen et al., 2005). This correlated to results obtained in this study, which also detected α-copaene in large and increasing concentrations during development of the fungal infection by *B. cinerea* in tomato plants.

Tiwari et al. (2020) proposes that α-copaene might be a general stress induced VOC, in their article on volatile biomarkers for quality management during postharvest storage of horticultural commodities (Tiwari et al., 2020). A remark is made during profiling of VOCs

that some fruits were observed to have brown and necrotic spots, which can also be an indication of fungal disease in the fruit tissue and thereby possibly a more specific response to pathogenic infection. Kasal (2016) also recognizes α-copaene as a stress induced volatile sesquiterpene, but similarly to Jansen et al. (2009), she found no increased emission of this compound when tomato plants were roughly handled. Both studies describe α-copaene as a VOC released as a plant-pathogen response and that a possible source might be the fungal infection by *B. cinerea* in tomato plants (Jansen et al. 2009; Kasal 2016).

The monoterpene β-phellandrene was also detected in large concentrations during experiments. Jansen et al. (2009) describes the high increase of this VOC as a general stress response of the plant when subjected to rough handling and following damage of the trichomes (Jansen et al. 2009). This might relate to results obtained in E3, as fungal infection might also cause damage to plant tissue including the trichomes. The plants of this experiment were not completely separated inside of the sampling boxes, and some touching of other plants or the walls of the boxes might have contributed to the release of this compound as well.

Cis-thujopsene was another possible biomarker candidate identified in E3. A review by Sankaran et al. (2010) describes an experiment where a VOC based profiling technique was applied by GC-MS to detect plant disease by non-destructive means. The findings presented by Li et al. (2009) mentioned thujopsene as a contributing compound in blueberries infected by *B. cinerea* and *Alternaria* spp. This might be indicative of a similar biological response of blueberry and tomato to the *B. cinerea* fungi. Cis-thujopsene was not detected in the vials containing only agar and *B. cinerea* in E1, and therefore it is likely to assume that this compound is somehow related to the plant's response to infection by this fungi (C. Li et al., 2009; Sankaran et al., 2010).

(+)-4-carene was also one of the dominant VOCs with increasing concentration during the disease development of *B. cinerea* in tomato leaves from E3. It is difficult to establish a connection between this compound and the pathogen related plant response. Some research indicate that 4-carene might be a general compound emitted by the tomato plants (Buttery et al., 1987), and especially as a VOC that increased with the age of the tomato plant, from young to older age (Zhang & Chen, 2009). Other research describe a connection between 4-carene

and VOCs released by the cultivation of different pathogenic fungal strains (Macías-Rubalcava et al., 2018).

Presence of the four candidate volatile biomarkers from E3 was not confirmed to be connected with the VOCs detected in E1, as no matches were found for the specific RT of each compound. It was assumed the RT would be corresponding if any matches were present, since parameters of the analytical procedures were constant for all performed experiments. This might indicate that (+)-4-carene, β-phellandrene, α-copaene and cis-thujopsene were indicative of a plant related response, and possibly VOCs released as an antimicrobial effect from the pathogen-plant interactions of *B. cinerea* infection in tomato plants.

4.4 Targeted analysis of VOCs produced by tomato plants infected by *B. cinerea*

In addition to the identification of possible biomarker compounds, a targeted approach was applied on the *B. cinerea* infected tomato plants to explore the possibility of detecting the same compounds in a larger volume of air which is more similar to actual greenhouse conditions. An assumption was made that with a selective ion monitoring (SIM) method in addition to regular scan, the VOCs could be detected in larger conditions. This was supported by the obtained results, as all the four compounds (+)-4-carene, β-phellandrene, α-copaene and cis-thujopsene were detected in low, but gradually increasing concentrations.

The control which was sampled from the room next door to the tomato plants, showed higher peaks for two of the compounds. The (+)-4-carene and cis-thujopsene peaks were higher for the control sample than the actual tomato plant sampling. The room used for sampling of the control had previously contained other plants, including cucumber plants quite recently. Some of these plants might have also released these exact VOCs, and possibly in high concentrations as the tomato plants were both low in number and small in size, compared to the cucumber plants.

5 Conclusion

Fungal pathogen attacks have a major impact on plant health, and biomarkers for disease monitoring could aid in the early detection of such diseases. This study aimed to investigate VOCs released during the infection of two typical greenhouse plants by related fungal pathogens. The establishment of a method for analysis of VOCs was performed, including the optimisation of several analytical parameters of HS-SPME-GC-MS instrumentation.

In the first experiment, cucumber and tomato fruits were infected with *P. aphanidermatum* and *B. cinerea*, respectively, and the released VOCs were analysed by GC-MS. In two other experiments, cucumber plants were infected by *P. aphanidermatum,* and tomato plants by *B. cinerea.* Static sampling was applied for the collection of volatiles from the cucumber plants, and dynamic sampling applied for the tomato plants. Untargeted profiling of the collected VOCs was used for these two experiments, which provided a global perspective of the plant systems under investigation. This can be effective for the detection and identification of large numbers of VOCs. In addition, the ability to detect VOCs using targeted analysis was tested on tomato plants.

The *Pythium*-induced root rot disease was not observed in inoculated cucumber plants, and the use of static sampling procedure resulted in a chromatogram with very few organic compounds. The implementation of dynamic sampling for the following experiment on grey mould disease in tomato plants, an infection caused by the fungal pathogen *B. cinerea*, allowed identification of four possible biomarker compounds. The monoterpenes (+)-4-carene and β-phellandrene, and sesquiterpenes α-copaene and cis-thujopsene, were all detected in gradually increasing concentrations throughout the experiment.

The volatile organic compound α-copaene seems likely to originate from a plant-pathogen interaction, and possibly from the specific infection of *B. cinerea* in tomato leaves. β-phellandrene is presumably induced as a more general stress response of the plant, as it can be emitted by other sources than fungal disease. Cis-thujopsene is another compound possibly released from the fungal interaction of *B. cinerea* on tomato plants. (+)-4-carene might be a compound constantly released from tomato plants and maybe other plants as well.

Based on the results of this research project, the two most likely biomarkers for the specific pathogen-plant interaction between tomato plants and the fungal pathogen *B. cinerea,* are α-copaene and cis-thujopsene. More research is needed to ultimately confirm this connection. These two relevant VOCs were emitted in different amounts, as the original concentration of α-copaene was larger than for cis-thujopsene. HS-SPME-GC-MS is a sensitive and suitable method for detection of VOCs released during fungal infection in plants. For future prospects, it would be interesting to perform more extensive and replicate experiments on *B. cinerea* infection in several plants including tomato, to find out if the two mentioned possible biomarkers are indeed specific for tomato plants. Other options for the continuation of this research could be to look for biomarkers specific to pathogenic infection in postharvest tomato fruits, to be able to detect disease at an early time-point and make this part of the production chain more effective and profitable.

6 References

Agilent Technologies. (2021). *What are the common contaminants in my GCMS*. Agilent Technologies, Inc. https://www.agilent.com/cs/library/Support/Documents/FAQ232%20F05001.pdf

Arthur, C. L., & Pawliszyn, J. (1990). Solid phase microextraction with thermal desorption using fused silica optical fibers. *Analytical Chemistry*, *62*(19), 2145–2148. https://doi.org/10.1021/ac00218a019

Baluška, F., & Ninkovic, V. (Eds.). (2010). *Plant Communication from an Ecological Perspective*. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-12162-3

Buettner, A. (Ed.). (2017). *Springer Handbook of Odor*. Springer International Publishing. https://doi.org/10.1007/978-3-319-26932-0

Buttery, R. G., Ling, L. C., & Light, D. M. (1987). Tomato leaf volatile aroma components. *Journal of Agricultural and Food Chemistry*, *35*(6), 1039–1042. https://doi.org/10.1021/jf00078a043

Cortina, P. R., Asis, R., Peralta, I. E., Asprelli, P. D., & Santiago, A. N. (2017). Determination of Volatile Organic Compounds in Andean Tomato Landraces by Headspace Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry. *Journal of the Brazilian Chemical Society*, *28*(1), 30–41. https://doi.org/10.5935/0103-5053.20160142

Creative Proteomics. (2021). *OPLS-DA Service*. Creative Proteomics. https://www.creative-proteomics.com/services/opls-da-service.htm

Deng, C., Zhang, X., Zhu, W., & Qian, J. (2004). Investigation of Tomato Plant Defence Response to Tobacco Mosaic Virus by Determination of Methyl Salicylate with SPME-Capillary GC-MS. *Chromatographia*, *59*, 263–268. https://doi.org/10.1365/s10337-003-0144-1

Ernst, W. H. O. (1999). Biomarkers in Plants. In D. B. Peakall, C. H. Walker, & P. Migula (Eds.), *Biomarkers: A Pragmatic Basis for Remediation of Severe Pollution in Eastern Europe* (pp. 135–151). Springer Netherlands. https://doi.org/10.1007/978-94-011-4550-3_10

Fan, S. (2018). *Introduction to Multivariate Analysis*.

Fang, Y., & Ramasamy, R. P. (2015). Current and Prospective Methods for Plant Disease Detection. *Biosensors*, *5*(3), 537–561. https://doi.org/10.3390/bios5030537

Gao, P., Qin, J., Li, D., & Zhou, S. (2018). Inhibitory effect and possible mechanism of a Pseudomonas strain QBA5 against gray mold on tomato leaves and fruits caused by Botrytis cinerea. *PLOS ONE*, *13*(1), e0190932. https://doi.org/10.1371/journal.pone.0190932

González-Mas, M. C., Rambla, J. L., Alamar, M. C., Gutiérrez, A., & Granell, A. (2011). Comparative Analysis of the Volatile Fraction of Fruit Juice from Different Citrus Species. *PLOS ONE*, *6*(7), e22016. https://doi.org/10.1371/journal.pone.0022016

Hermansen, A., Stensvand, A., & Bøvre, J. (2018, February 22). *Gråskimmel* [Encyclopedia]. NIBIO Plantevernleksikonet. https://www.plantevernleksikonet.no/l/oppslag/466/

Hermansen, A., Sundheim, L., & Toppe, B. (2011, July 12). *Pythiumråte* [Encyclopedia]. NIBIO Plantevernleksikonet. https://www.plantevernleksikonet.no/l/oppslag/1160/

Islam, M. T., Rahman, M. M., Pandey, P., Boehme, M. H., & Haesaert, G. (Eds.). (2019). *Bacilli and Agrobiotechnology: Phytostimulation and Biocontrol: Volume 2*. Springer International Publishing. https://doi.org/10.1007/978-3-030-15175-1

Jansen, R. M. C., Hofstee, J. W., Wildt, J., Verstappen, F. W. A., Bouwmeester, H. J., Posthumus, M. A., & Henten, E. J. V. (2009). Health monitoring of plants by their emitted volatiles: Trichome damage and cell membrane damage are detectable at greenhouse scale. *Annals of Applied Biology*, *154*(3), 441–452. https://doi.org/10.1111/j.1744-7348.2008.00311.x

Jansen, R. M. C., Hofstee, J. W., Wildt, J., Verstappen, F. W. A., Bouwmeester, H., & van Henten, E. J. (2009). Induced plant volatiles allow sensitive monitoring of plant health status in greenhouses. *Plant Signaling & Behavior*, *4*(9), 824–829. https://doi.org/10.4161/psb.4.9.9431

Jansen, R. M. C., Wildt, J., Hofstee, J. W., Bouwmeester, H. J., & van Henten, E. J. (2010). Plant Volatiles: Useful Signals to Monitor Crop Health Status in Greenhouses. In F. Baluška & V. Ninkovic (Eds.), *Plant Communication from an Ecological Perspective* (pp. 229–247). Springer. https://doi.org/10.1007/978-3-642-12162-3_13

Kasal, T. (2016). *Use of Biogenic Volatile Organic Compounds to assess the Health Status of Tomato Plants (Solanum Lycopersicum) cv. "Moneymaker"* [PhD in Agriculture, Rhenish Friedrich Wilhelm University of Bonn]. https://d-nb.info/109509906X/34

Lee, J. H. J., Jayaprakasha, G. K., Avila, C. A., Crosby, K. M., & Patil, B. S. (2019). Metabolomic studies of volatiles from tomatoes grown in net-house and open-field conditions. *Food Chemistry*, *275*, 282–291. https://doi.org/10.1016/j.foodchem.2018.09.091

Li, C., Krewer, G., & Kays, S. J. (2009). Blueberry Postharvest Disease Detection Using an Electronic Nose. *2009 Reno, Nevada, June 21 - June 24, 2009*. 2009 Reno, Nevada, June 21 - June 24, 2009. https://doi.org/10.13031/2013.27360

Li, J., Fu, Y., Bao, X., Li, H., & Zuo, J. (2019). Optimization of solid phase microextraction combined with gas chromatography-mass spectrometry (GC-MS) to analyze aromatic compounds in fresh tomatoes. *Journal of Food Biochemistry*, *43*(10). https://doi.org/10.1111/jfbc.12858

López-Gresa, M. P., Lisón, P., Campos, L., Rodrigo, I., Rambla, J. L., Granell, A., Conejero, V., & Bellés, J. M. (2017). A Non-targeted Metabolomics Approach Unravels the VOCs Associated with the Tomato Immune Response against Pseudomonas syringae. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.01188

Macías-Rubalcava, M. L., Sánchez-Fernández, R. E., Roque-Flores, G., Lappe-Oliveras, P., & Medina-Romero, Y. M. (2018). Volatile organic compounds from Hypoxylon anthochroum endophytic strains as postharvest mycofumigation alternative for cherry tomatoes. *Food Microbiology*, *76*, 363–373. https://doi.org/10.1016/j.fm.2018.06.014

Mandal, A. (2021). *What is a Biomarker?* News-Medical.Net. https://www.news-medical.net/health/What-is-a-Biomarker.aspx

Martinelli, F., Scalenghe, R., Davino, S., Panno, S., Scuderi, G., Ruisi, P., Villa, P., Stroppiana, D., Boschetti, M., Goulart, L. R., Davis, C. E., & Dandekar, A. M. (2015). Advanced methods of plant disease detection. A review. *Agronomy for Sustainable Development*, *35*(1), 1–25. https://doi.org/10.1007/s13593-014-0246-1

MetaboAnalyst. (2021). *Metabolomic Data Analysis with MetaboAnalyst 5.0*. https://www.metaboanalyst.ca

NIBIO. (2021, June 18). *Særheim*. Norwegian Institute of Bioeconomy Research. https://www.nibio.no/en/about-eng/addresses/saerheim

NIST. (2021). *Chemical Name Search*. National Institute of Standards and Technology: NIST Chemistry WebBook; National Institute of Standards and Technology. https://webbook.nist.gov/chemistry/name-ser/

Paré, P. W., & Tumlinson, J. H. (1999). Plant Volatiles as a Defense against Insect Herbivores. *Plant Physiology*, *121*(2), 325–332. https://doi.org/10.1104/pp.121.2.325

Petrasch, S., Silva, C. J., Mesquida-Pesci, S. D., Gallegos, K., van den Abeele, C., Papin, V., Fernandez-Acero, F. J., Knapp, S. J., & Blanco-Ulate, B. (2019). Infection Strategies Deployed by Botrytis cinerea, Fusarium acuminatum, and Rhizopus stolonifer as a

Function of Tomato Fruit Ripening Stage. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.00223

Petrisor, C., & Paica, A. (2019). Overview on utilities and analysis techniques of organic volatile compounds (VOCs) produced by fungi belonging to Trichoderma spp. *The Publishing House of the Romanian Academy*, *21*(2), 91–98.

Piasecka, A., Kachlicki, P., & Stobiecki, M. (2019). Analytical Methods for Detection of Plant Metabolomes Changes in Response to Biotic and Abiotic Stresses. *International Journal of Molecular Sciences*, *20*(2). https://doi.org/10.3390/ijms20020379

Quintana-Rodriguez, E., Rivera-Macias, L. E., Adame-Alvarez, R. M., Torres, J. M., & Heil, M. (2018). Shared weapons in fungus-fungus and fungus-plant interactions? Volatile organic compounds of plant or fungal origin exert direct antifungal activity in vitro. *Fungal Ecology*, *33*, 115–121. https://doi.org/10.1016/j.funeco.2018.02.005

Rambla, J. L., Alfaro, C., Medina, A., Zarzo, M., Primo, J., & Granell, A. (2015). Tomato fruit volatile profiles are highly dependent on sample processing and capturing methods. *Metabolomics*, *11*(6), 1708–1720. https://doi.org/10.1007/s11306-015-0824-5

Rambla, J. L., López-Gresa, M. P., Bellés, J. M., & Granell, A. (2015). Metabolomic Profiling of Plant Tissues. In J. M. Alonso & A. N. Stepanova (Eds.), *Plant Functional Genomics: Methods and Protocols* (pp. 221–235). Springer. https://doi.org/10.1007/978-1-4939-2444-8_11

Ray, M., Ray, A., Dash, S., Mishra, A., Achary, K. G., Nayak, S., & Singh, S. (2017). Fungal disease detection in plants: Traditional assays, novel diagnostic techniques and biosensors. *Biosensors and Bioelectronics*, *87*, 708–723. https://doi.org/10.1016/j.bios.2016.09.032

Sánchez-Ortiz, B. L., Sánchez-Fernández, R. E., Duarte, G., Lappe-Oliveras, P., & Macías-Rubalcava, M. L. (2016). Antifungal, anti-oomycete and phytotoxic effects of volatile organic compounds from the endophytic fungus Xylaria sp. Strain PB3f3 isolated from Haematoxylon brasiletto. *Journal of Applied Microbiology*, *120*(5), 1313–1325. https://doi.org/10.1111/jam.13101

Sankaran, S., Mishra, A., Ehsani, R., & Davis, C. (2010). A review of advanced techniques for detecting plant diseases. *Computers and Electronics in Agriculture*, *72*(1), 1–13. https://doi.org/10.1016/j.compag.2010.02.007

Sankaranarayanan, A., & Amaresan, N. (2020). *Pythium* [Elsevier's premier platform of peer-reviewed literature]. ScienceDirect. https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/pythium

Sigma-Aldrich Co. (2004). Solid Phase Microextraction Troubleshooting Guide. *Bulletin 928A*, 12.

Skoog, D. A., Holler, F. J., & Crouch, S. R. (2017). *Principles of Instrumental Analysis*. Cengage Learning.

Souza Silva, É. A., Saboia, G., Jorge, N. C., Hoffmann, C., dos Santos Isaias, R. M., Soares, G. L. G., & Zini, C. A. (2017). Development of a HS-SPME-GC/MS protocol assisted by chemometric tools to study herbivore-induced volatiles in Myrcia splendens. *Talanta*, *175*, 9–20. https://doi.org/10.1016/j.talanta.2017.06.063

Teledyne Tekmar. (2012). *Comparison of Static and Dynamic Headspace for Volatile Organic Compounds in Orange Juice*. https://www.teledynetekmar.com/resources/Application%20Notes/Headspace%20VOCs%20in%20Orange%20Juice.pdf

Thelen, J., Harbinson, J., Jansen, R., Straten, G. V., Posthumus, M. A., Woltering, E. J., & Bouwmeester, H. J. (2005). The sesquiterpene α-copaene is induced in tomato leaves infected by Botrytis cinerea. *Journal of Plant Interactions*, *1*(3), 163–170. https://doi.org/10.1080/17429140600968177

Tipler, A. (2020). An Introduction To Headspace Sampling In Gas Chromatography Fundamentals And Theory. *PerkinElmer*, 35.

Tiwari, S., Kate, A., Mohapatra, D., Tripathi, M. K., Ray, H., Akuli, A., Ghosh, A., & Modhera, B. (2020). Volatile organic compounds (VOCs): Biomarkers for quality management of horticultural commodities during storage through e-sensing. *Trends in Food Science & Technology*, *106*, 417–433. https://doi.org/10.1016/j.tifs.2020.10.039

Tromelin, A., Andriot, I., & Guichard, E. (2006). Protein–flavour interactions. In *Flavour in Food* (pp. 172–207). Elsevier. https://doi.org/10.1533/9781845691400.2.172

Tsugawa, H., Cajka, T., Kind, T., Ma, Y., Higgins, B., Ikeda, K., Kanazawa, M., VanderGheynst, J., Fiehn, O., & Arita, M. (2015). MS-DIAL: Data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nature Methods*, *12*(6), 523–526. https://doi.org/10.1038/nmeth.3393

Weingart, G., Kluger, B., Forneck, A., Krska, R., & Schuhmacher, R. (2011). Establishment and Application of a Metabolomics Workflow for Identification and Profiling of Volatiles from Leaves of Vitis vinifera by HS-SPME-GC-MS: Metabolomics Based

Identification of VOC in Vitis Leaves by SPME-GC-MS. *Phytochemical Analysis*, *23*(4), 345–358. https://doi.org/10.1002/pca.1364

Zainol Hilmi, N. H., Idris, A. S., & Mohd Azmil, M. N. (2019). Headspace solid-phase microextraction gas chromatography-mass spectrometry for the detection of volatile organic compounds released from Ganoderma boninense and oil palm wood. *Forest Pathology*, *49*(4), e12531. https://doi.org/10.1111/efp.12531

Zhang, P., & Chen, K. (2009). Age-Dependent Variations of Volatile Emissions and Inhibitory Activity Toward Botrytis cinerea and Fusarium oxysporum in Tomato Leaves Treated with Chitosan Oligosaccharide. *Journal of Plant Biology*, *52*(4), 332–339. https://doi.org/10.1007/s12374-009-9043-9

Appendix A

Literature review on sampling of VOCs by SPME

Table 6: Literature overview of SPME applied in VOC associated plant research. ID: inner diameter, IE: ionisation energy, MA: mass acquisition range, NIST: National Institute of Standards and Technology mass spectral library, PCA: principal component analysis, DA: discriminant analysis, HCA: hierarchical cluster analysis.

Plant species	**Tissues**	**Collection method details**	**Type of VOCs collected**	**SPME type**	**Analytical method**	**Method details**	**Analytical software**	**References**
Oil palm *(Elaeis guineensis Jacq.)*	Wood shavings inoculated with the fungal pathogen *Ganoderma boninense*	HS-SPME with manual sampling. Pre-incubation: 30 min at 40°C Extraction: 15 min at 40°C Desorption: "rapidly" at 250°C	Alcohols, alkanes, volatile acids, ketones, aldehydes, esters, sesquiterpenes, polycyclic hydrocarbon groups.	50/30 µm DVB/CAR/PDMS Conditioning of fibre: 30 min at 250°C	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Carrier gas: Helium Flow rate: 1 ml/min Temperature: Init. 45°C for 1 min, 10°C/min to 260°C, 260°C for 30 min, transfer line 260°C IE: 70 eV MA range: 20-400 m/z	Data analysis by *Agilent Enhanced MSD ChemStation (Version E.02.00.493)* Identification by *NIST library* Multivariate statistical analysis by *Microsoft Excel* Chemometric analysis by *MetaboAnalyst 4.0*	(Zainol Hilmi et al., 2019)
Tomato (*Solanum lycopersicum* L. and *Solanum pimpinellifolium*)	Red ripe fruit	HS-SPME with automatic sampling. 1 ml processes sample transferred to 10 ml vial with silicone septum. Pre-incubation: 10 min at 50°C, 500 rpm shaking speed. Extraction: 35 min at 50°C, 250 rpm shaking speed Desorption: 1 min at 250°C	Aldehydes, alcohols, monoterpenes, sesquiterpenes, ketones, esters, acids, aromatic compounds, thiazole, and more	50/30 µm DVB/CAR/PDMS Bakeout of fibre: 3 min at 250°C	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Carrier gas: Helium 5.0 ultrapure Flow rate: 1 ml/min Temperature: 35°C for 5 min, 3°C/min to 45°C, 1.5°C/min to 50°C, 50°C for 1.5 min, 3°C/min to 68°C, 68°C for 2 min, 3°C/min to 131°C, 131°C for 1 min, 10°C/min to 250°C, 250°C for 2.93 min MS trap 200°C, manifold 100°C and transfer line 230°C Runtime per chromatographic analysis: 58 min IE: 70 eV MA range: 33-300 amu, 1 scan per second	Mass spectra analysed by *Varian MS Workstation (Version 6.6)* Univariate and multivariate (PCA and DA) statistical analysis by *Infostat*	(Cortina et al., 2017)
Tomato *(Solanum lycopersicum* L. cv. Moneymaker)	Fruit	HS-SPME with manual sampling. 2 ml of processed sample transferred to 22 ml vial. Pre-incubation: 10 min at 50°C, 300 rpm shaking speed Extraction: 10 min at 50°C, 300 rpm shaking speed Desorption: 1 min at 250°C, splitless injection	Aldehydes, ketones, alcohols, alkene, thiazole	65 µm PDMS/DVB Bakeout of fibre: 5 min at 250°C	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Carrier gas: Helium Flow rate: 1.2 ml/min Temperature: 40°C for 3 min, 3°C/min to 60°C, 6°C/min to 160°C, 12°C/min to 260°C, 260°C for 5 min MS trap 200°C, manifold 100°C and transfer line 230°C IE: 70 eV MA range: 35-300 m/z	Chromatograms and spectra recorded with *GC/MS TurboMass (version 5.0, Perkin Elmer)* PCA analysis by *SIMCA-P 10.0 (Umetrics)* HCA analysis by *Acuity 4.0 (Axon Instruments)*	(Rambla, Alfaro, et al., 2015)
Tomato *(Solanum lycopersicum* cv. Fendi)	Ripe fruit	HS-SPME Internal standard: 1-heptanol No added salt (NaCl tested)	Aldehydes, hydrocarbons, alcohols, ketones, oxygen-containing heterocyclic compounds, esters, sulfur-and	85 µm CAR/PDMS	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Carrier gas: Helium Flow rate: 1.3 ml/min Temperature:	Identification by *NIST*	(J. Li et al., 2019)

		Pre-incubation: Extraction: 60°C Desorption:	nitrogen-containing heterocyclic compounds			35°C for 0 min, 3°C/min to 180°C, 15°C/min to 230°C, 230°C for 2 min IE: 70 eV MA range: 50-500 amu		
Sweet orange *(Citrus sinensis* L. Osb.) Clemenules *(Citrus clementine* Hort. Ex Tan.) Two hybrids: Fortune (*C. grandis* x *C. tangerine)* and Chandler pummelo (*C. grandis* x *C. grandis)*	Ripe fruit	HS-SPME with automatic sampling. 10 ml of fruit juice sample in 22 ml vial. Pre-incubation: 10 min at 50°C Extraction: 20 min at 50°C Desorption: 1 min at 250°C, splitless injection	Esters, aldehydes, alcohols, monoterpene hydrocarbons, ketones, sesquiterpene hydrocarbons, monoterpene cyclic ethers, furans, aromatic hydrocarbons	50/30 µm DVB/CAR/PDMS	GC-MS	Capillary column: 60 m x 0.25 mm ID x 1 µm film thickness Carrier gas: Helium Flow rate: 1.2 ml/min Temperature: 40°C for 2 min, 5°C/min to 250°C, 250°C for 5 min IE: 70 eV, source temperature 230°C MA range: 35-220 m/z, seven scans per second	Chromatograms and spectra recorded with *Enhanced ChemStation for GC-MS (Agilent)* Identification by *NIST (2005)* PCA analysis by *SIMCA-P version 11 (Umetrics)* HCA analysis by *Acuity 4.0 (Axon Instruments)* Pearson correlation coefficients calculated with *SPSS version 15.0*	(González-Mas et al., 2011)
Tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker)	Leaves inoculated with the fungal pathogen *Botrytis cinerea*	SPME portable field sampler (1 cm coating length, 100 µm film thickness) Leaf placed in purified water inside petri dish, glass lid with rubber septum. Extraction: room temperature for 2 h Dynamic sampling: volatiles collected on cartridges, HS air drawn through the tubes using air sampler with flow 100ml/min +/- 5%, sample collection times of 30 or 60 min Desorption: 5 min at 250°C, splitless injection	Mostly monoterpenes and sesquiterpenes	100 µm PDMS for first sampling Dynamic sampling: 90 mg Tenax TA 20/30 mesh cartridges	GC-FID GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Carrier gas: Nitrogen Temperature: 60°C for 4 min, 6°C/min to 220°C IE: 70 eV, source temperature 230°C MA range: 24-300 m/z, seven scans per second	Data collection by *Peaknet (Dionex Corp.)* Statistical analysis by *Unscrambler version 9.5, PeakFIt version 4.12* and *SPSS*	(Thelen et al., 2005)
Tomato (not specified)	Seedlings	HS-SPME, type not mentioned. 5.0 l glass headspace bottle used Pre-incubation: 10 min at 25°C Extraction: 15 min at 25°C Desorption: 2 min at 270°C, splitless injection	Aromatic hydrocarbon, monoterpenes, ester, sesquiterpene	100 µm PDMS Bakeout of fibre: 270°C for 10 min	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Carrier gas: Helium (99.999%) Flow rate: 1 ml/min Temperature: 80°C for 2 min, 15°C/min to 300°C, 300°C for 10 min, transfer line temperature 280°C IE mode used MA range: 41-500 amu, seven scans per second	Identification by *NIST Mass Spectral Search Program*	(Deng et al., 2004)
Strawberry (not specified)	Fruit	HS-SPME with automatic sampling 900 µl of homogenised mixture transferred to 10 ml vial Pre-incubation: 10 min at 50°C, agitation at 500 rpm Extraction: 30 min at 50°C with agitation Desorption: 1 min at 250°C, splitless mode	Alcohols, esters, ketones, aldehydes, acids, monoterpenes, sesquiterpenoids, hydrocarbons	65 µm DVB/PDMS Bakeout of fibre: 5 min at 250°C	GC-MS	Capillary column: 60 m x 0.25 mm ID x 1.0 µm film thickness Carrier gas: Helium 5.0 Flow rate: 1.2 ml/min Temperature: 40°C for 3 min, 5°C/min to 250°C, 250°C for 5 min, transfer line temperature 260°C, ionisation source 230°C, MS quadrupole 150°C Chromatographic time: 50 min IE: 70 eV MA range: 35-250 m/z, six scans per second	Untargeted analysis by *MetAlign*	(Rambla, López-Gresa, et al., 2015)
Tomato (eight	Fruit	HS-SPME with automatic sampling.	Alcohols, aldehydes, esters, fatty acids, furans,	50/30 µm CAR/PDMS/DVB	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness	Data recorded and processes by *Xcalibur v. 2.0.7.*	(Lee et al., 2019)

different varieties)		2 g of sample was put into 20 ml glass vial with chemical liquids. Vortexed for 1 min and sonicated for 30 min at room temp. Pre-incubation: 2 min at 60°C with continuous agitation (5 s/min) Extraction: 45 min at 60°C with continuous agitation (5 s/min) Desorption: 2 min at 225°C, splitless mode	hydrocarbons, ketones, and sulphur compounds			Carrier gas: Helium Flow rate: 1 ml/min Temperature: 40°C for 1 min, 10°C/min to 90°C, 3°C/min to 175°C, 35°C/min to 230°C, 230°C for 2 min, transfer line temperature 250°C, ion source 280/180°C Chromatographic time: 38 min IE: 70 eV MA range: 40-450 m/z, 11.5 scans per second	*(Thermo Fisher Scientific)* Spectra compared in *Wiley 8* and *NIST 05 mass spectral library* Univariate analysis by *SPSS v. 23* Multivariate analysis by *Excel* and *MetaboAnalyst 4.0*	
Punchberry tree (*Myrcia splendens)*	Leaves and galls	HS-SPME with manual sampling. 25 mg of sample and 2µl of internal standard was transferred into 20 ml vials. Pre-incubation: 30 min at 30°C without agitation Extraction: 15 min Desorption: 15 min at 240°C	Alcohols, aldehydes, esters, ketones, phenol derivatives, monoterpenes and sesquiterpenes	85 µm DVB/CAR/PDMS, 65 µm DVB/CAR/PDMS and 50/30 µm DVB/PDMS	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Carrier gas: Helium (99.999%) Flow rate: 1 ml/min Temperature: 35°C for 1 min, 3°C/min to 140°C, 140°C for 1 min, 5°C/min to 220°C, 220°C for 3 min, transfer line temperature 250°C, ion source 200°C, quadrupole 150°C IE: 70 eV MA range: 35-350 m/z	Identification by *NIST 08* HCA and PCA analysis using *XLStat2011 (Addinsoft) for Microsoft Excel*	(Souza Silva et al., 2017)
Grapevine (*Vitis vinifera* cv. Pinot Noir clone 18 Gm*)*	Leaves	HS-SPME with automatic sampling. 105 mg of homogenised leaf sample transferred to 20 ml vial. Pre-incubation: 30 min at 90°C without agitation Extraction: 60 min at 90°C Desorption: 2 min at 250°C, splitless mode	Terpenes, alcohols, aldehydes, ketones and aromatic compounds	50/30 µm DVB/CAR/PDMS Conditioning of fibre: 1 h at 270°C Bakeout of fibre: 10 min at 270°C (for each extraction)	GC-MS	Capillary column: 30 m x 0.25 mm ID x 0.25 µm film thickness Liner: 1.5 mm HS-liner (Gerstel) Carrier gas: Helium 5.0 Flow rate: 1 ml/min Temperature: 35°C for 2 min, 5°C/min to 260°C, 260°C for 5 min, transfer line temperature 270°C, ion source 230°C, quadrupole 150°C IE: 70 eV MA range: 35-500 m/z, 3 scans per second	Data evaluation and deconvolution by *AMDIS v. 2.65* Mass spectra compared with *Wiley/NIST 08 MS library*	(Weingart et al., 2011)

Appendix B

MS-DIAL alignment results for tomato fruit samples infected with *B. cinerea* in E1

Table 7: List of all deconvoluted peaks in the alignment results of experiment E1, and their corresponding retention times, retention indexes and quant masses.

Alignment ID	Average RT (min)	Average RI	Quant mass
0	2.205	669.35	44.00
1	2.348	672.86	45.07
2	2.367	673.33	44.00
3	2.399	674.11	49.00
4	2.431	674.89	58.00
5	2.434	674.99	70.05
6	2.448	675.32	43.08
7	2.498	676.55	44.00
8	2.505	676.72	67.00
9	2.528	677.29	40.00
10	2.534	677.44	55.00
11	2.58	678.56	45.00
12	2.594	678.9	46.00
13	2.631	679.82	78.90
14	2.634	679.88	44.00
15	2.676	680.91	42.00
16	2.684	681.11	59.01
17	2.736	682.39	75.00
18	2.802	684	45.00
19	2.911	686.68	43.00
20	2.938	687.33	82.00
21	2.981	688.38	45.01
22	2.981	688.39	69.05
23	3.029	689.56	40.00
24	3.04	689.85	43.00
25	3.063	690.41	71.01
26	3.077	690.74	44.00
27	3.084	690.91	41.00
28	3.13	692.03	49.00
29	3.143	692.36	83.00
30	3.147	692.45	57.00
31	3.148	692.48	46.90
32	3.19	693.51	43.00
33	3.205	693.89	40.00
34	3.228	694.45	44.00
35	3.277	695.66	84.80
36	3.316	696.61	66.98
37	3.423	699.23	82.00

38	3.445	699.77	53.00
39	3.47	700.38	41.05
40	3.657	704.97	44.00
41	3.665	705.15	41.00
42	3.669	705.27	43.00
43	3.738	706.95	56.03
44	3.767	707.67	91.00
45	3.785	708.11	147.00
46	4.046	714.51	44.00
47	4.106	715.98	57.00
48	4.151	717.08	96.00
49	4.173	717.62	81.00
50	4.178	717.73	55.00
51	4.212	718.56	41.00
52	4.22	718.77	59.00
53	4.374	722.54	57.06
54	4.473	724.97	43.00
55	4.847	734.14	43.00
56	4.893	735.28	41.02
57	4.999	737.88	77.00
58	5.079	739.84	55.01
59	5.165	741.94	57.08
60	5.247	743.94	84.00
61	5.316	745.63	71.00
62	5.597	752.54	56.00
63	5.929	760.67	55.00
64	6.25	768.53	56.00
65	6.254	768.64	42.98
66	7.213	792.14	41.00
67	7.22	792.32	57.00
68	7.345	795.01	43.00
69	8.124	811.36	207.00
70	8.44	817.43	56.05
71	10.746	861.76	56.03
72	11.248	871.42	70.05
73	11.743	880.93	43.00
74	16.421	954.08	83.10
75	18.4	983.13	43.00
76	18.61	986.21	81.00
77	18.668	987.06	53.00
78	18.933	990.94	133.70
79	18.967	991.44	192.93
80	18.968	991.45	248.95
81	18.988	991.76	107.00
82	29.285	1145.81	191.00
83	29.298	1146.02	73.00
84	34.161	1232.8	115.00
85	34.17	1232.98	91.00

86	34.197	1233.51	92.00
87	36.054	1269.72	147.00
88	38.032	1309.19	73.00
89	38.051	1309.62	133.05
90	38.069	1310.01	189.10
91	38.152	1311.8	175.10
92	40.549	1363.69	107.00
93	40.553	1363.78	122.00
94	40.555	1363.82	175.10
95	45.147	1479.2	206.90
96	45.163	1479.64	73.02
97	45.17	1479.83	146.98
98	48.658	1632.94	73.00
99	48.659	1632.98	220.97
100	50.836	1775.89	72.98
101	52.533	1903.36	72.97
102	54.002	1939.37	72.98
103	55.297	1971.13	72.99
104	56.689	2005.26	72.90