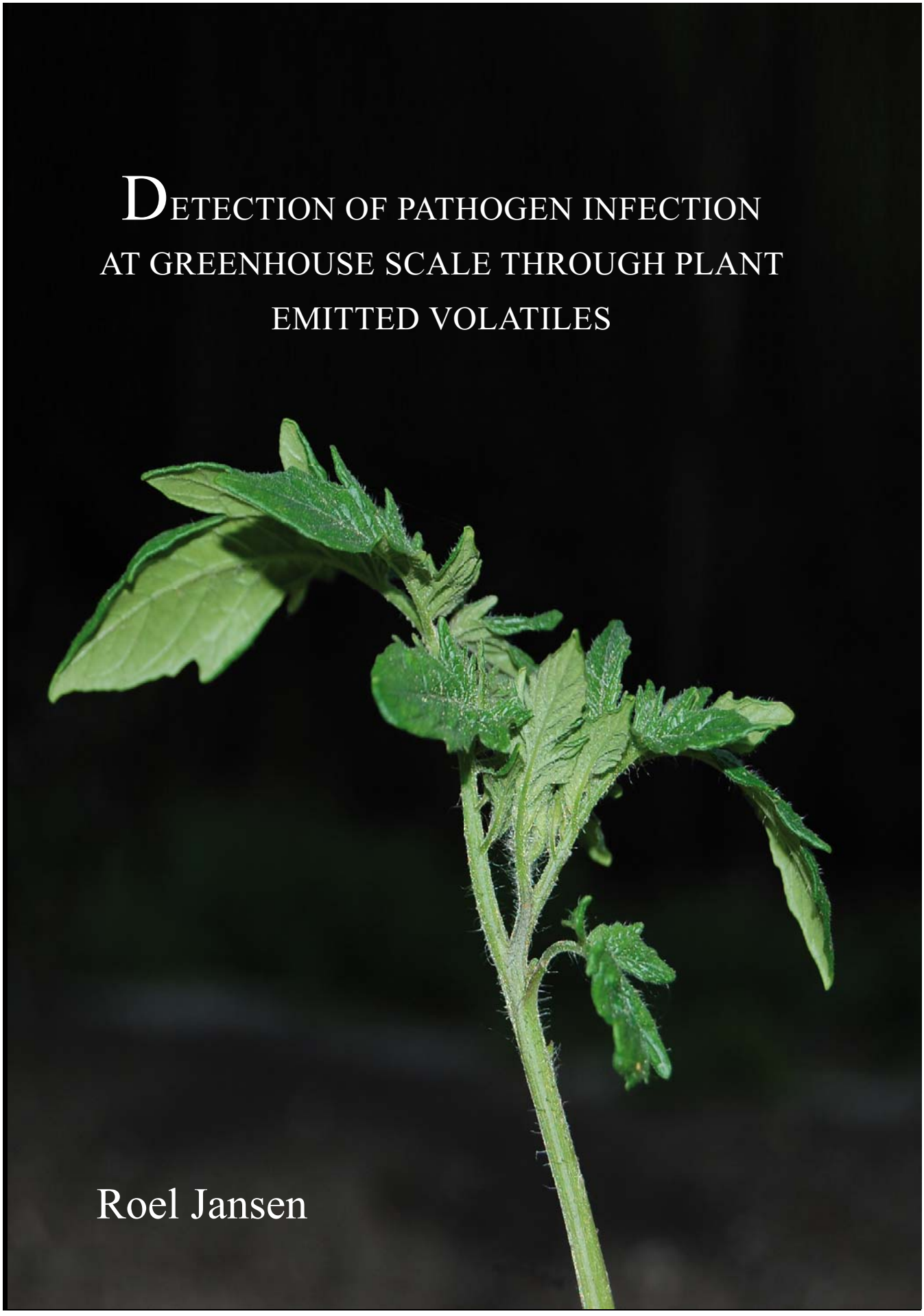
Detection of pathogen infection at greenhouse scale through plant emitted volatiles



Roel Jansen

DETECTION OF PATHOGEN INFECTION AT GREENHOUSE SCALE THROUGH PLANT EMITTED VOLATILES

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DETECTION OF PATHOGEN INFECTION AT GREENHOUSE SCALE THROUGH PLANT EMITTED VOLATILES

RUDOLF MARTINUS CORNELIS JANSEN

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VOORWOORD

In dit voorwoord beschrijf ik de aanleiding voor het tot stand komen van dit proefschrift. Ik geef je een kort terugblik op wat vooraf ging en ik neem de tijd om een aantal mensen te bedanken.

Mijn belangstelling voor natuur- en scheikunde werd gewekt door de kadootjes die ik vroeger kreeg: technisch lego, een scheikundedoos waarmee je leuke proefjes kon doen en een doos met elektraonderdelen waarmee je bijvoorbeeld een alarmsysteem voor je slaapkamer kon maken. Ook mijn belangstelling voor biologie werd jong gewekt, het lijkt me dat de vele wandelingen door het bos, het vissen en het spelen op de boerderij hier sterk aan hebben bijgedragen.

Een studie met veel aandacht voor biologie, natuurkunde, of scheikunde? Dat was de vraag waarmee ik worstelde bij mijn keuze voor hoger onderwijs. Deze drie vakken waren favoriet, al was handenarbeid natuurlijk ook erg leuk. Ik heb destijds met mijn vader drie hogescholen bezocht. Een school in Den Bosch ‘waar men iets deed met chemie’, een school in Velp ‘waar met zich bezig hield met de levende natuur’ en een school in Arnhem ‘voor technische zaken’. Na niet al te lang te hebben nagedacht koos ik voor werktuigbouwkunde aan de Hogere Technische School (HTS) te Arnhem. Hier kon je –volgens mijn vader– in ieder geval altijd wel een boterham mee verdienen in de toekomst, een argument dat ik destijds als onbelangrijk afdeed.

Mijn belangstelling voor biologie bleef. Mijn eerste HTS stage liep ik dan ook bij het Instituut voor Milieu en Agritechniek (IMAG) te Wageningen. Hier werd onderzoek verricht naar de ontwikkeling van een oogstrobot voor komkommers. Een prachtige stage waarbij natuur en techniek samenkwamen. Ook mijn tweede stage periode heb ik te danken aan het IMAG. Ik heb namens dit instituut onderzoek verricht aan dierlijke tractie voor bodembewerking in Zambia. Ook hier kwam natuur en techniek bij elkaar.

Na mijn studie werktuigbouwkunde het ik een aantal keer gesolliciteerd maar al snel werd duidelijk; ik wilde niet de hele dag technische tekeningen maken, zittend achter een computerscherm. Daarop besloot ik het internet af te struinen. Mogelijk kon ik met mijn HTS diploma aansluiten op een andere opleiding waarbij techniek en natuur centraal staan. De

opleiding Landbouwtechnische Wetenschappen aan de Wageningen Universiteit leek mij ideaal. Vakken over planten, over dieren, vaak met een flinke scheut techniek overgoten. Dat deze universiteit dicht bij Arnhem lag vond ik bovendien een groot voordeel omdat ik mij met deze stad vertrouwd voelde en daarom niet opnieuw wilde verhuizen. De opleiding Landbouwtechnische Wetenschappen werd voor een groot deel verzorgd door de leerstoelgroep Agrarische Bedrijfstechnologie. Toen Wim Huisman, destijds verbonden aan deze leerstoelgroep tijdens een college vroeg: "Wie wil er na zijn studie promotieonderzoek verrichten?" vloog mijn hand als enige omhoog. Onderzoeker, dat klonk toch een beetje magisch.

Nu, vijf jaar later weet ik, onderzoek kent inderdaad zijn magische momenten. Dit zijn momenten waarbij mensen op je pad komen, juist als je ze nodig hebt, resultaten verschijnen die je totaal niet verwacht en ideeën opborrelen die je nooit eerder hebt gehad. Daarnaast kent onderzoek ook saaie, vermoeiende, en drukke momenten. Juist dan heb je hulp nodig.

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Sache dienlich ist, wenn man seinem Gegenüber gegenläufige Ansichten auf möglichst taktvolle Art und Weise zu verstehen gibt. Vielen Dank aber vor Allem fur die Hilfe bei der Ausführung und Interpretation der vielen Messungen im Labor des Instituts „Phytosphere" in Jülich (Deutschland).

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Roel

CONTENTS

CHAPTER 1

General introduction

SETTING THE STAGE: THE WORLD'S GREENHOUSE PRODUCTION AREAS

The world's greenhouse industry will attain ever greater importance since the cultivation of greenhouse crops offers an excellent opportunity to grow high-quality products in large quantities on a small surface area.

Where are the world's main greenhouse production areas? In order to evaluate published statistics, definitions are important. Unfortunately, there are few precise ones for "greenhouse". In some cases, very simple plastic or shade-cloth structures are called "greenhouses". For example, there is a reported 40.000 ha. of "greenhouse" vegetable production in Almeria, Spain (Costa, 2001). Most of this production is in very simple flat-roofed structures covered with plastic. Mexico currently is producing in an estimated 2.200 ha. of more advanced, passively ventilated, high-tunnel structures (Steta, 2004). These are unheated, plastic covered metal structures, with insect netting side walls, and have computerized irrigation and fertilization systems. The high end of the greenhouse structure spectrum is found in The Netherlands (Hickman, 2009). Here, more than 10.000 ha. of greenhouses are located which are mainly used to produce vegetables, flowers and pot plants. These greenhouses are primarily high technology metal structures covered with glass, with computer controlled environments. Due to the unclear definition of "greenhouse", the estimates of the total worldwide greenhouse production area varies widely. An approximation of greenhouse production areas world-wide is presented in Table 1.1.

Table 1.1 Greenhouse production areas world-wide (Giacomelli *et al.*, 2008).

	Plastic greenhouses and large plastic tunnels[ha]	**Glasshouses [ha]**	**Total [ha]**
Western Europe	140.000	29.000	169.000
Eastern Europe	25.000	1.800	26.800
Afrika	27.000	600	27.600
Middle East	28.000	13.000	41.000
North America	9.850	1.350	11.200
Central/South America	12.500	0	12.500
Asia/Oceania	450.000	2.500	452.500
Total	692.350	48.250	740.600

Greenhouses in the Netherlands are ‘big business’. In 2005, the Dutch greenhouse horticultural sector had a total added value of €4.6 billion, representing more than 20% of the added value of the Dutch agricultural complex based on domestic raw materials (Breukers *et al.*, 2008). Most of the greenhouse production areas in The Netherlands are used for food production in which tomato, cucumber and sweet pepper are the important crops. New, high yielding varieties contribute to improved incomes in the greenhouse production. Another way to improve income in to increase the scale in order to achieve greater efficiency and lower cost. For that reason, greenhouse industry moves toward large-scale systems while small greenhouses are being closed (Breukers *et al.*, 2008). However, increases in scale tends to raise new problems. One particular problem is addressed in this thesis.

PROBLEM DESCRIPTION

The cultivation of crops in large-scale greenhouses is characterized by the monoculture of high-value crops at high plant density throughout the entire year. These conditions lead to increased productions per surface area. However, the year-round production of one single, high-density crop, also provides excellent circumstances to establish and disperse pathogen infections in the greenhouse (van Lenteren, 2000). At present, the initial introduction and subsequent dispersal of pathogen infections is mainly controlled by the preventative application of chemicals. However, chemical control methods have several important disadvantages. First, the human intake of pesticide residues on fruits and vegetables cause an increasing concern among consumers (Juraska *et al.*, 2007). Second, chemical control methods are not sustainable since pathogens may quickly become resistant to the active ingredients (Elad and Evensen, 1995). Third, the exposure of chemicals has a negative effect on greenhouse workers health and also pollute the environment (Gil and Sinfort, 2005, Wang *et al.*, 2008).

In addition to chemical control, the threat of infection requires the regular human inspection in order to anticipate and do curative control. The inspections must be accurate in large-scale greenhouses since inaccurate inspections then allow pathogen infections to disperse rapidly over long distances which in turn result in large economic losses. These accurate, on-site inspections of crops are time-consuming and requires skilled personnel which in-turn leads to high costs. As a result, greenhouse managers become increasingly dependent on automation

to limit the demand for manual labour that carries out these on-site inspections. Thus, expansion and intensification of greenhouse industry have increased the demand for an automated system to detect pathogen infections at an early stage.

Imaging techniques provide an important contribution to current research related to early and non-invasive disease detection in greenhouse horticulture (Boissard *et al.*, 2008, Chaerle and van der Straeten, 2001). However, the disadvantages are that they can only provide information on outer canopy layers and they can only detect a late stage of infection when visual symptoms have developed. A more adequate method for the detection of pathogen infection should provide information on both inner and outer canopy layers and should warn for problems in an early stage before disease symptoms appear. Such a method would facilitate immediate actions and prevent further spread by controlling the problem right at the source.

OBJECTIVE

A novel approach to discover a pathogen attack would be the detection of specific volatile organic compounds (VOCs) emitted from pathogen infected plants. This idea is based upon the numerous laboratory studies which revealed that pathogen infections have an effect on the volatile blend released by plants (Cardoza *et al.*, 2002, Deng *et al.*, 2004b, Shulaev *et al.*, 1997, Vuorinen *et al.*, 2007). This approach is non-invasive. But, in contrast to imaging, it has the potential to provide information on both inner and outer canopy layers and it has the potential to detect problems before visual symptoms appear. Based on this prospective, the main research objective was formulated.

The main research objective of this study was to investigate whether plant emitted VOCs can be used to detect a pathogen infection in a large-scale greenhouse.

SCOPE AND DELIMITATION

This study is focused on the detection of the grey mould disease in tomato plants. The motivation to select tomato as our model plant was threefold. First, greenhouse production of tomato is an economically important industry worldwide. Second, tomato production suffers from yield losses caused by several diseases. Third, the tomato is a well established model in plant pathogen-interactions studies (Arie *et al.*, 2007).

Grey mould is caused by the fungal pathogen *Botrytis cinerea*. Our motivation to select this pathogen was based on two aspects. First, this pathogen is one of the most comprehensively studied, and thus extensive body of literature exists. Second, this pathogen is a well-known cause for considerable damage in a broad range of plant species including tomato (Elad and Stewart, 2004).

The picture in Fig. 1.1 shows an everyday situation in Dutch greenhouse tomato production: a stem infection of tomato with *B. cinerea*. An early warning would help greenhouse personnel to discover such infection at an early stage and allows them to cut it out before the whole stem is damaged and save the plant from dying.

Fig. 1.1 Stem infection of tomato (*Lycopersicon esculentum*) with the necrotrophic pathogen *Botrytis cinerea*. Photo: Rudi Aerts

RESEARCH QUESTIONS

The detection of a *B. cinerea* infection through plant emitted VOCs requires knowledge about *B. cinerea* induced emissions of VOCs from tomato. Consistent and unique emissions are required if these emissions would be the sole source of information to detect a *B. cinerea* infection. However, besides the effect of a *B. cinerea* infection, probably also other factors affect the emission rates of VOCs from tomato. One may think of other stressors, but also environmental factors such as an increase in temperature or light intensity. Perhaps, crop operations such as harvest also affect the emission rates of VOCs from tomato plants. These factors should be considered to determine the reliability of VOC emissions as sole information source. The final piece of the puzzle is a careful look at the *B. cinerea*-induced concentrations of VOCs in large scale greenhouses. They are essential parameters to consider when evaluating analytical instruments for sensing VOCs in large-scale tomato producing greenhouses.

These thoughts and facts from literature lead to the following research questions:

1. What is the effect of a *B. cinerea* infection on the emission of VOCs from tomato?
    a. What are the emission of VOCs from healthy, undisturbed tomato?
    b. What are the emission of VOCs from tomato upon a *B. cinerea* infection?
    c. Is there a relation between the severity of infection and the emission of VOCs from tomato?
2. Are *B. cinerea*-induced emissions of VOCs from tomato specific for the infection with this pathogen?
    a. What are the effects of biotic and abiotic stresses on the emission of VOCs from tomato?
    b. What are the effects of environmental factors on the emission of VOCs from tomato?
    c. What are the effects of crop operations on the emission of VOCs from tomato?
3. Are *B. cinerea*-induced concentrations of VOCs detectable in large-scale greenhouses?
    a. What are the *B. cinerea*-induced concentrations of VOCs in large-scale greenhouses?
    b. Are there analytical instruments available which are able to detect these *B. cinerea*- induced concentrations of VOCs in a large-scale tomato production greenhouse?

OUTLINE OF THIS THESIS

The work presented in this thesis is outlined below.

CHAPTER 1 is the general introduction. It gives an overview of the world's greenhouse production areas, it introduces the problem and it presents arguments why there is need to study new ways for detecting plant pathogens in greenhouse horticulture.

CHAPTER 2 describes the initial laboratory experiments which were undertaken to get familiar with equipment and to study the emissions of VOCs from non infected as well as *B. cinerea* infected tomato leaves.

CHAPTER 3 describes the experiments to reveal changes in emissions of VOCs from whole intact tomato plants upon infection with *B. cinerea*. In these experiments, we monitored the emissions of tomato plants semi-continuously with a time interval of about one hour. Furthermore, VOC emissions were quantified.

CHAPTER 4 is based on experiments performed in a small-scale greenhouse. In these experiments, two types of plant damage were applied to study whether they are detectable in a greenhouse through emissions of VOCs by the plants. Furthermore, the effects of picking fruits and removing side-shoots on volatile emissions were studied.

CHAPTER 5 reports on a particular experiment performed in a small-scale greenhouse. This experiment was conducted to see whether plant emitted VOCs can be used to detect a *B. cinerea* infection in a small tomato producing greenhouse.

In CHAPTER 6, a model is described to characterize the source/sink behaviour of plant emitted volatiles in a greenhouse This model was used to determine whether VOCs can be used to detect *B. cinerea* infections in large-scale tomato producing greenhouses.

The research described in CHAPTER 7 aimed at studying whether complex data obtained during measurements on VOCs can be automatically processed in order to determine the concentrations of them.

In CHAPTER 8, the results obtained in this study are summarised and placed in a broader perspective. Furthermore we give recommendations for further developments and an outlook on the possibilities for crop health monitoring based on plant emitted VOCs in the future.

CHAPTER 2

The effect of *Botrytis cinerea* on the emission of volatile organic compounds from detached leaves

Published as:

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INTRODUCTION

Botrytis cinerea is a major fungal pathogen which is known to cause grey mould in at least 235 host plant species, including greenhouse crops like tomato (Jarvis, 1977). Therefore, about $20m / year is spent worldwide on chemical control of *B. cinerea* in greenhouses (Elad and Stewart, 2004). This control depends upon first identifying the presence of a pathogen, a procedure which is currently dependent upon skilled personnel and is not automated. Current trends in glasshouse technology and management are founded on increasing automation and improved sensors, and this should also apply to disease or pathogen detection within the crop. These developments, and a drive to uncover how *B. cinerea* colonizes tomato tissue, are the motivation for this work.

Plants produce a wide range of volatile and nonvolatile secondary metabolites (Croteau *et al.*, 2000). In the past these compounds received little attention from ecologists and evolutionary biologists as it was thought that they were waste products of primary metabolism. Today, secondary metabolites are widely studied (Dixon, 2001, Theis and Lerdau, 2003) and, because they can play a role in protecting plants against a wide variety of microorganisms (viruses, bacteria, and fungi) (Wink, 1988) they are increasingly the focus of breeding programmes including metabolic engineering (Aharoni *et al.*, 2005). The monoterpenes (C_{10}) and sesquiterpenes (C_{15}) are secondary metabolite terpenoids that are volatile at temperatures commonly encountered under field and glasshouse conditions, thus contributing to the volatile organic compounds (VOCs) produced by plants. Monoterpenes are known as components of the volatile essences of flowers and of the essential oils of herbs and spices. Sesquiterpenes are also found in essential oils and, in addition, numerous sesquiterpenes act as phytoalexins, antibiotic compounds produced by plants in response to microbial challenge, and as antifeedants that discourage herbivory (for review see Croteau *et al.*, 2000, Stoessl *et al.*, 1976). Volatile terpenoids that are induced by insect herbivory have also been shown to be implicated in the attraction of natural enemies of these insects (Kappers *et al.*, 2005).

Given their role in pest and disease response and their volatile nature it has been suggested that measuring the type and amounts of terpenoids in the air of a glasshouse might be a means of monitoring the health of glasshouse crops (Baratto *et al.*, 2005). This idea is particularly relevant to future closed greenhouse designs which will have no free ventilation and within which VOCs could accumulate, thus making detection more feasible. An implication of this

is, of course, that these concentrated VOCs will have an increased effect on crops when they act as messengers of some kind (Wheeler *et al.*, 2004). In research on other stresses, like herbivore damage (Ament *et al.*, 2004) and mechanical damage (Maes, 2002), the stressed tomato plants produced a different spectrum of VOCs than control plants. The hypothesis tested here is that *B. cinerea* infected tomato plants produce a different spectrum of VOCs than healthy tomato plants. Measuring changes in the VOC fingerprint would therefore allow discrimination between a healthy tomato plant and an infected tomato plant, which could eventually lead to a disease detection system. The main research question to be answered is whether we can discriminate between a control plant and a plant infected with *B. cinerea* based on the VOC fingerprint. The second research question is to identify the VOCs that allow for this discrimination to be made, because these VOCs would be candidates for designing a disease detection system.

MATERIALS AND METHODS

Plant material

Tomato seedlings (*Lycopersicon esculentum* Mill. cv. Moneymaker) were grown in 17 cm pots in potting compost (Lentse Potgrond, Lent, The Netherlands) in a greenhouse (natural light) with day and night temperatures of 20°C and 18°C, respectively. The relative humidity was always 70%. Prior to the experiments tomato plants were transferred to a climate room at 25±1°C and 65±10% relative humidity. Irradiance was supplied by fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands) for 15 h / 9 h light/dark photoperiod with a photosynthetically active radiation of 120 μmol m^{-2} s^{-1} at leaf height. The light intensity was measured with a Li-250 photosynthetically active quantum flux meter (LiCor, Lincoln, Nebraska, USA).

Inoculation procedures

Botrytis cinerea strain (B0510) was cultured on malt extract agar (CM0059, Oxoid, Basingstoke, UK) at 25±1°C in 9-cm Petri dishes in the dark. Plates were inoculated in a laminar flow hood with a small droplet of spore suspension. After four days, cultures were exposed for 24 h to near-UV light to stimulate sporulation. One to two weeks after near-UV stimulation, conidia were harvested from the sporulating mycelium by washing with sterile water, containing 0.05% Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany), and rubbing

the mycelium. Conidia were filtered through glass wool, washed three times by centrifuging (8 min, 800 rpm, 20°C) and resuspended in sterile water. The spores were finally resuspended in an inoculum buffer prepared as described by Benito *et al.* (1998) at a density of 10^9 L^{-1}. The inoculum buffer consisted of filter-sterilized water (0.2 µm, FP30 / 0,2 CA-S, Schleicher & Schuell, Dassel, Germany), supplemented with Gamborg's B5 medium (Duchefa Biochemie bv, Haarlem, The Netherlands), 10 mM glucose and 10 mM potassium phosphate (pH 6). The conidia concentration was counted under a light microscope with the use of a haemocytometer. The suspension was pre-incubated for 2-3 h, with occasional shaking by hand. Leaves of 4- and 7-week-old tomato plants were inoculated on the upper surface with eight 2 ml droplets on each of 8 leaves per plant. After inoculation, the plants were placed inside a transparent plastic tent for 16 h at 95% RH to stimulate infection. Relative humidity was checked with a humidity sensor (HMP 233, Vaisala, Helsinki, Finland) and the air was humidified periodically with an ultrasonic humidifier (572011, Conrad Electronic GmbH, Hirschau, Germany) attached to a time switch. After a 16-h infection period, the relative humidity was reduced to 85%; this humidity level accelerated the spreading of infection. Control plants were treated in exactly the same way, except that the inoculation droplets contained no spores. The infected and control plants were separated from each other during the infection process to prevent contamination of the control plants.

Sampling of volatiles

Six hours before headspace sampling, inoculated plants were taken out of the humidifier to allow droplets on the leaf surfaces to evaporate. Plants were placed in a separate part of the climate room to prevent cross-contamination by other plants. Leaves (1±0.5 g fresh weight) were carefully removed from the plant with a scalpel and placed in a glass Petri dish (300 ml). Care was taken not to damage the trichomes on the leaf. The petiole of the detached leaf was placed in purified water (Milli Q, Millipore, Bedford, MA, USA) to prevent dehydration, which itself could have induced VOC release. The Petri dish was immediately closed by a glass lid containing rubber septum (7824, Alltech, Deerfield, IL, USA).

For quantification, volatiles were statically collected on polydimethylsiloxane (PDMS) fibres, which were housed in a solid phase microextraction (SPME) portable field sampler (1 cm coating length, 100 µm film thickness, Supelco, Bellefonte, PA, USA). Prior to use, fibres were conditioned in a gas chromatograph (GC) injection port at 250°C and subjected to two

30-min GC runs to purge any contaminating volatiles. Sampling was carried out by inserting the SPME piercing needle through the septum and exposing the fibre to the headspace for 2 h. There were 5 replicate samples of both the infected and control leaves. For identification of VOCs, dynamic sampling had to be performed to increase absolute amounts of the volatiles absorbed. In the dynamic sampling procedure the volatiles were collected on cartridges containing 90 mg Tenax TA (20/30 mesh, Chrompack, Walnut Creek, CA, USA) and head-space air was drawn through the tubes using a portable battery-operated air sampler with constant flow of 100 ml min^{-1} ±5% (Ametek / du Pont de Nemours & Co., type Alpha-2, DEHA International, Huizen, The Netherlands). The sample collection times were 30 or 60 min and the flow-rate was checked with a soap bubble meter. Both infected and control leaves were sampled in three replicates. All glassware used in the experimental set-up was thoroughly cleaned with detergent and water and afterwards heated overnight in an oven (Binder, South Korea) at 25°C to remove any contaminating volatiles.

Analysis of volatiles

Volatiles were thermally desorbed for 5 min in a Varian 3400 CX gas chromatograph equipped with a flame ionization detector (Varian, Walnut Creek, CA, USA). The injections were made in splitless mode with a 0.75-mm inner diameter glass inlet liner (Supelco, Bellefonte, PA, USA). The GC-FID was operated with an injector temperature of 250°C and a detector temperature of 300°C. Compounds were separated on a capillary DB-5 column (30 m × 0.25 mm ID, film thickness 0.25 μm; J&W Scientific, Folsom, California) at 40°C for 5 min after which the temperature was increased at 8°C min^{-1} to 225°C; this temperature was then maintained for 5 min. Nitrogen was used as the carrier gas. After 5 min the split was opened at 1:50. Data were collected by a personal computer running the Peaknet software package (Dionex Corporation, Sunnyvale, CA, USA). An *n*-alkane mix (C_{10}-C_{17}) was run through the column to provide a scale for the calculation of the retention index (RI). For most VOCs the RI is known (Adams, 2001). Therefore we could identify the region in the chromatogram that was interesting for further analysis. For the VOCs from tomato plants the RI was between 1000 and 1700. For GC-MS analysis, volatiles were transferred to a capillary DB-5 column (60 m × 0.25 mm ID, film thickness 0.25 μm) mounted in a Varian 3400 GC and connected to a Finnigan MAT 95 mass spectrometer. The oven temperature was set at 60°C for 4 min and then programmed to 220°C at a rate of 6°C min^{-1}. The mass spectrometer was operated in the 70 eV electron impact ionization mode and scanning was from mass 24 - 300 at 0.7 sD^{-1}.

Compounds were identified by comparing the mass spectra and RI with those in the Adams collection (Adams 2001) and in the Wageningen Mass Spectral Library.

Statistical analysis

A fundamental problem in GC-FID data analysis is finding a suitable representation of this high-dimensionality data. Linear transformation is often chosen for dimension reduction in multivariate data (Hyvarinen, 1999). Principal component analysis (PCA) (Joliffe, 1986) is a well known linear transformation method for reducing the dimensionality of metabolomics datasets (Hendriks *et al.*, 2005, Jansen *et al.*, 2004, Norwich Research Park, 2006). PCA defines a model of multivariate data. This model is a lower dimensional subspace that explains the direction of maximum variance in the original data matrix. Our multivariate data is a matrix with 18000 columns with retention variables measured on a set of 10 rows of samples (called objects). These samples are the chromatograms from the headspace of control and infected leaves. The first few principal components (PCs) explain most of the variance in the original data. Between chromatograms the retention time of identical compounds can differ; this is a problem inherent to GC analysis (Hendriks *et al.*, 2005, Pate *et al.*, 1998, Rohrback and Ramos, 2003, Tomasi *et al.*, 2004). Therefore, prior to PCA, the reduced chromatograms were pre-processed with the correlation optimized wrapping (COW) alignment algorithm (Tomasi *et al.*, 2004). COW is a piecewise or segmented data pre-processing method aimed at aligning a sample data vector to a reference vector by allowing limited changes in segment lengths on the sample vector (Tomasi *et al.*, 2004). The chromatogram with the highest number of compounds was chosen as reference, as suggested by Tomasi *et al.* (2004). The COW algorithm was used as implemented in COWTool (v1.1, BioCentrum-DTU, Technical University of Denmark). The parameters were: segment length (m) was 25, so the number of segments (N) was therefore 720, and slack (t) was 2. After alignment the data was loaded into a multivariate statistics software package (Unscrambler v9.5, Camo A/S, Trondheim, Norway) for PCA analysis. First, the date was mean-normalized, which is a standard procedure when performing PCA on chromatography data, then the PCA was conducted for nine principal components. Mean normalization consists of dividing each chromatogram by its average peak height. Peak areas for the GC-FID data were calculated using the Gaussian function in PeakFit v4.12 (Seasolve Software Inc, Framingham, MA, USA) for peak fitting and calculation of peak area. The data was baseline corrected with the function Best 2D. GC-FID and GC-MS peak areas were first normalized by the area percent

method. Thus the total area of the identified peaks in each chromatogram was 100%. Then an independent *t*-test (SPSS Inc, Chicago, Il, USA) was performed to test whether the mean peak area of the identified peaks of healthy control leaves and infected leaves are statistically different from each other.

RESULTS

The penetration of the host tissue by *B. cinerea* is a process that can be divided into three sequential phases (Benito *et al.*, 1998): Killing of the host tissue, formation of primary lesion, and formation of secondary lesion. After five days infected leaves had severe secondary lesions, whereas the control leaves had no visual lesions. These leaves were used for determination and identification of volatiles. In Fig. 2.1 and Fig.2.2, raw GC-FID chromatograms obtained from head-space samples of a healthy, control leaf and an infected leaf are shown. Clear differences between the control and infected leaf chromatograms are evident. An infected leaf emits a greater amount of VOCs, and the large peak at 20 min in infected leaves (Fig. 2.2) is nearly absent in control leaves (Fig. 2.1).

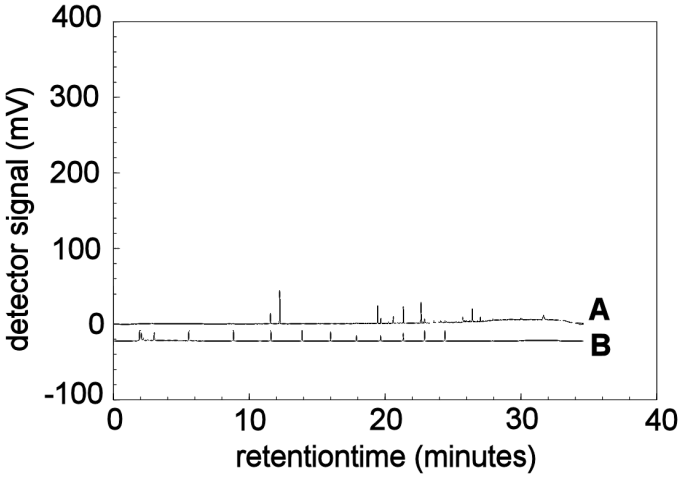

Fig. 2.1 Raw GC-FID chromatogram of the headspace of a healthy detached leaf (A), a C_7-C_{17} *n*-alkane mix (B) is shown below as a reference.

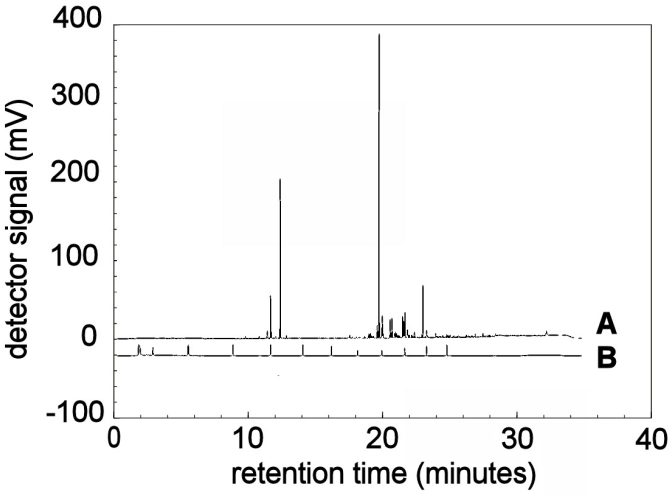

Fig. 2.2 Raw GC-FID chromatogram of the headspace of an infected detached leaf (A), a C_7-C_{17} *n*-alkane mix (B) is shown below as a reference.

The region between C_{10} and C_{17} contains the VOCs known to be emitted by tomato plants; therefore, for further analyses the data was reduced to the region between 10 and 25 min, which is equivalent to 18,000 data points.

Principal component analysis

Principal components build a link between samples (chromatograms) and variables (detector signal at the different retention times) by means of scores and loadings. The scatter plot of the scores on the first two principal components shows clustering of the five control and five infected samples (Fig. 2.3).

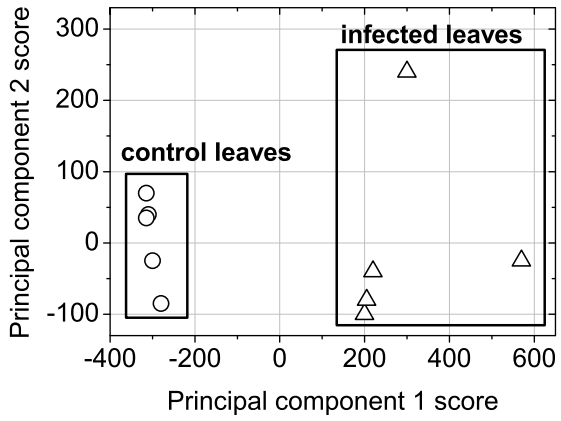

Fig. 2.3 PCA score scatter plot of the first two principal components, after alignment of GC-FID chromatograms of the headspace of five control tomato leaves and five infected tomato leaves.

The first two principal components explained 96% of the variance present in the data set. The loading plot is used to identify which compounds are most responsible for separating of the 10 chromatograms into two groups. In Fig. 2.3, it is shown that infected leaves have a positive first principal component and healthy control leaves have a negative first principal component. The loading plot of the first principal component is shown in Fig. 2.4. If a variable has a high positive loading, it means that all chromatograms with a positive first principal component have higher than average values for that value. Thus the high positive loading in Fig. 2.4 of the variable at data point 11500 explains why all infected leaf chromatograms have a positive first principal component. The data point at 11500 is equivalent to a retention time of 20 min.

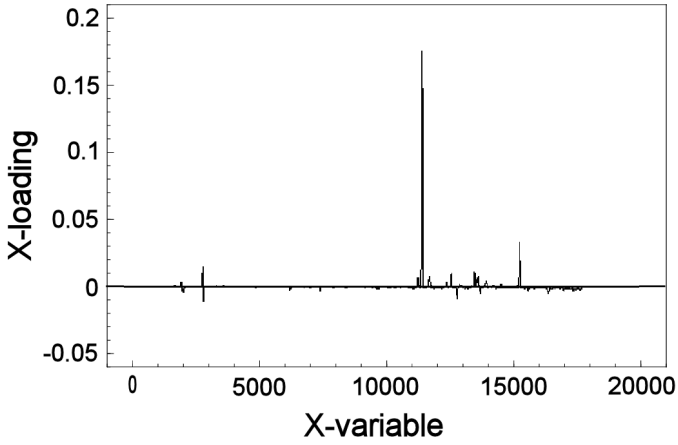

Fig. 2.4 PCA loading plot of the first principal component, after alignment of GC-FID chromatograms of the headspace of five control tomato leaves and five infected tomato leaves.

Identification of the volatile fingerprint

The VOCs detected by GC-MS were mostly mono and sesquiterpenes. In Table 2.1, the VOCs present in control and infected samples are shown (excluding all Tenax-derived impurities). Six out of the 27 different VOCs that were detected occurred in each chromatogram. The dominant peak at 20 min in the GC-FID chromatograms of the headspace of infected leaves could now be identified as α-copaene. Injection of an authentic α-copaene standard (27814, Fluka, Buchs, Switzerland) into the GC-FID showed an identical retention time to the major peak located at 20 min in the GC-FID chromatograms of headspace air from infected leaves. Cyclosativene and β-cubebene have a retention index close to α-copaene, as

shown in Table 2.1, but different mass spectra. To be sure, the mass spectrum of the *α*-copaene peak was carefully checked for any deviations in intensity or extra peaks which could point to a co-eluting other compound.

Table 2.1 All detected volatiles emitted by tomato leaves by GC-MS analysis. Compounds are ordered according to their calculated retention index (RI). Samples of type I are peak areas from chromatograms of the headspace of infected leaves, samples of type C are peak areas from chromatograms of the headspace of control leaves. The compounds that are marked with an A occurred in each sample. Peak areas marked with B also contain impurities and peak areas marked with C also contain other terpenes. Trace levels are marked 'tr.', 'n.d.' means not detected in the mass spectra and '-' means not in this chromatogram.

	Sample	1	2	3	4	5	6
	Type	I	C	I	C	I	C
Compound	**RI**	**Peak area**					
α-pinene[A]	939	3	4	40	15	24	4
myrcene	991	-	-	22	9[B]	-	-
2-carene[A]	1002	21	100	419	158	119	24
sabinene		-	21	-	-	-	-
α-phellandrene	1003	-	4	6	4	-	-
3-carene		-	-	1	-	-	-
p-cymene[A]	1025	1	4	28	6	18	2
limonene[A]	1029	8	58	199	64	71	15
β-phellandrene[A]	1030	53	330	1340	394	405	43
β-ocimene		-	-	-	-	-	2
methyl salicylate		-	4	-	-	tr.[B]	2
γ-terpinene	1060	-	-	3	-	-	-
DMNT[1]		-	-	1	-	-	-
cyclosativene	1371	3	-	34	2	12	-
α-copaene[A]	1377	37	14	656	48	208	tr. (0.6)
β-cubebene	1388	-	-	12	n.d.	4	-
β-elemene[2]		-	-	tr.	2[C]	-	-

sativene	1392	-	-	7	n.d.	3	-
β-ylangene	1420	-	-	tr.	-	tr.	-
β-caryophyllene	1421	-	-	13	3	4	-
α-gurjunene		-	-	-	4	1	3
β-copaene	1432	-	-	29	6	11	-
γ-muurolene	1480	2	-	33	2	11	-
germacrene D	1485	-	-	4	1	2	-
α-muurolene	1500	-	-	7	-	3	-
δ-cadinene	1523	-	-	tr.	n.d.	tr.	-
TMTT[3]	1579	-	7	11	11	23	4
Number of identified VOCs		8	10	23	16	19	10

[1] 4,8-dimethyl-1,3,7-nonatriene (DMNT)

[2] *β*-elemene is likely to indicate of the presence of germacrene A, which becomes thermally rearranged to *β*-elemene in the injection port of the GC-MS (de Kraker *et al.*, 1998).

[3] (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)

Statistical analyses of GC-FID and GC-MS data

For proper statistical tests of the possible significance of differences in volatile production, normalization of the peak area is needed. In dynamic sampling this is done by injection of a standard with a known concentration into the sampling tube. In static sampling with SPME, which was done for acquiring the GC-FID data, this is not practical (Zabaras and Wyllie, 2001). In SPME analysis headspace concentrations are calculated by injection of a monoterpene and sesquiterpene solution of which the concentration is known. The GC-FID should be equipped with a special 100% PDMS column (Maes, 2002). This column was however not available in our laboratory. Normalization by the area percent method is then the most convenient method for normalization.

Peak areas of identified peaks in the GC-FID and GC-MS chromatograms were calculated and a *t*-test was done on these peak areas to discriminate between healthy control leaves and infected leaves. The GC-FID peak area of two major tomato volatile compounds (Ament *et al.*, 2004, Buttery *et al.*, 1987, Maes, 2002) (11.70 and 12.40 min) previously identified by GC-MS as 2-carene and *β*-phellandrene, and the peak (described here) at 20 min, which was

identified as α-copaene, were used. These three peaks were also identified by calculating their retention index. In Table 2.2, the result of that test is shown. All three compounds were found to be significantly different between control and infected samples ($p \leq 0.05$). The peak areas of the five peaks that were present in all GC-MS chromatograms were tested for statistical significance. Table 2.3, shows the results of that test. In this case, 2-carene and α-copaene were found to be significantly different between control and infected samples ($p \leq 0.05$).

Table 2.2 Volatiles emitted by tomato leaves, *t*-test on the GC-FID data. Compounds are ordered according to their calculated Retention index (RI). The values indicate the means of the normalized peak areas in percentages and standard deviation of each treatment group. The *p*-values have been calculated with an independent *t*-test ($n = 5$). Independent samples, normality, and equal variances were assumed. The compounds that are marked with an asterisk (*) were found to be significantly different ($p \leq 0.05$) for infested leaves versus control leaves.

Compound	RI	Control leaves [%]	Infected leaves [%]	*p* value
2-carene	1001	14.12 ± 5.20	4.04 ± 2.48	0.04*
β-phellandrene	1031	83.59 ± 4.37	14.53 ± 10.67	0.00*
α-copaene	1377	2.29 ± 2.70	81.43 ± 13.12	0.00*

Table 2.3 Volatiles emitted by tomato leaves, *t*-test on the GC-MS data. Compounds are ordered according to their calculated Retention index (RI). The values indicate the means of the normalized peak areas in percentages and standard deviation of each treatment group. The *p*-values have been calculated with an independent *t*-test ($n = 3$). Independent samples, normality, and equal variances were assumed. The compounds that are marked with an asterisk (*) were found to be significantly different ($p \leq 0.05$) for infested leaves versus control leaves.

Compound	RI	Control leaves [%]	Infected leaves [%]	*p* value
α-pinene	939	2.50 ± 1.88	2.26 ± 0.69	0.85
2-carene	1002	23.25 ± 3.74	15.59 ± 1.50	0.03*
p-cymene	1025	1.31 ± 0.83	1.33 ± 0.70	0.97
limonene	1029	12.55 ± 3.93	7.44 ± 0.95	0.09
β-phellandrene	1030	56.92 ± 8.10	46.99 ± 3.53	0.12
α-copaene	1377	3.48 ± 3.23	26.39 ± 3.20	0.00*

DISCUSSION

This study was undertaken to identify volatile compounds associated to Botrytis infection of tomato plants. The production of volatiles may be used as a non-destructive measurement of plant health. Here we have shown that infected tomato leaves produce increased amounts of VOCs and that *α*-copaene is the most prominent volatile induced by the tomato leaf-*B. cinerea* interaction. This conclusion is based upon the principal components analysis of the GC-FID data which identified a peak with an RI of 20 min as the major contributor to the dominant axis separating healthy and infected leaves, and the identification of this 20-min peak as *α*-copaene using GC-MS. The limonene peak (RI 1029), one of the other major compounds observed in the GC-MS chromatograms (Table 2.1), could not be identified in the GC-FID chromatograms. This is possibly because this compound is masked in the *β*-phellandrene peak (RI 1030), which has an almost identical RI. VOCs marked with an 'A' in Table 2.1 were also found in studies on herbivore stressed tomato plants by Dicke *et al.* (1998), Farag *et al.* (2002), Thaler *et al.* (2002), Kant *et al.* (2004), and Maes (2002). Notably, the production of *α*-copaene was not found to increase significantly in any of these studies. This suggests that *α*-copaene production may be associated with fungal infections in tomato. So, *α*-copaene may be at the least specific for infections and possibly specific for fungal infections or even those of Botrytis.

It is known that other compounds are formed during the interaction between tomato leaves and *B. cinerea*, especially nitric oxide (NO), hydrogen peroxide (H_2O_2) (Mahalingam and Fedoroff, 2003) and ethylene (C_2H_4) (Díaz *et al.*, 2002). These are of low molecular weight and, in the case of NO and H_2O_2, they are highly reactive with fast degradation times. The release of NO and H_2O_2 is related to programmed cell death, which occurs in response to pathogens and various abiotic stresses (Levine, 2004). It is possible to detect C_2H_4, and (in spite of their reactive nature) NO and H_2O_2 online (Conrath *et al.*, 2004, Cristescu *et al.*, 2002). These volatiles are, however, not suitable for monitoring fungal infection because they are emitted after most biotic and abiotic stresses. Botrydial ($C_{17}H_{26}O_5$) is a bicyclic sesquiterpene produced in plant tissues infected by *B. cinerea* (Deighton *et al.*, 2001). The vapour pressure of this compound is, however, too low for it to make a significant contribution to the VOCs produced by the infected leaves at ambient temperatures, and it could, therefore, not be detected.

Ideally the identification of plant disease marker compounds should be correlated with measurements of the enzymes responsible for the synthesis of the compounds and the genes which encoded the enzymes. By this more complete analysis of volatile production, and its control, can the association of a specific volatile with a particular stimulus be better understood (Dicke *et al.*, 2003). However the reactions catalyzed by many of these enzymes are complex and diverse, often generating one to upwards 30 reaction products (Steele *et al.*, 1998). In the case of volatile production by Arabidopsis flowers, only two enzymes are responsible for the complex mixture of sesquiterpenes (Tholl *et al.*, 2005). To our knowledge, no α-copaene synthase has been so far identified (Cane P, Tholl D, Croteau R, Fraga D, personal communication), and until this enzyme (or its gene) has been identified a more complete analysis of the control of α-copaene synthesis will not be possible. Nonetheless the physiological data presented here do strongly suggest that α-copaene is a good candidate molecule for the detection of Botrytis (and possibly other fungal) infections in crops.

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CHAPTER 3

The effect of *Botrytis cinerea* on the emission of volatile organic compounds from whole plants

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INTRODUCTION

Gray-mold rot or Botrytis blight, caused by the widespread necrotrophic fungus *Botrytis cinerea*, affects most vegetable and fruit crops. This pathogen cause severe losses to greenhouse-grown tomato all over the world (Elad and Stewart, 2004). It affects leaves, petioles, stems and fruits and consequently the quantity and quality of the harvested produce. A common management strategy to control *B. cinerea* in greenhouse tomato production is to spray fungicides. But fungicide spraying is costly and may have consequences in terms of health risk for the greenhouse employees, consumer risks due to residues on the produce and pollution of the environment. Fungicide use can be reduced if *B. cinerea* infestation is detectable at a very early stage. Then local treatment of the infested plants can be employed instead of full field treatment. However, early detection of *B. cinerea* infection is quite a challenge. Today, in horticultural practice, detection of *B. cinerea* is based on visual inspection. Visual inspection is time consuming and therefore expensive. It requires a considerable amount of expertise, is subjective, and fails in case plants carry latent infections which effects are not yet visible on the plant surface. With PCR and the ELISA test (Lievens *et al.*, 2006) *B. cinerea* can be detected at an early stage, but these procedures are invasive, time consuming, expensive, and demand careful sampling methods when investigating whole populations of plants.

The present study focuses on an alternative approach for non-invasive early detection of *B. cinerea* based on the detection of volatile organic compounds (VOCs) emitted by infected plants. There are a limited number of studies in which pathogen-infected plants have been shown to release VOCs that differ from those released by uninfected plants. For example, silver birch (*Betula pendula*) infected with the fungus Marssonina betulae emitted an increased amount of VOCs (Vuorinen *et al.*, 2007); an increase in emissions was also reported upon infection of tobacco plants (*Nicotiana tabacum*) with tobacco mosaic virus (Shulaev *et al.*, 1997); and a similar increase was observed upon infection of tobacco plants with the bacterial pathogen *Pseudomonas syringae* (Heiden *et al.*, 2003). These plant emissions might serve as signals for detection of infected plants at the greenhouse scale.

De Moraes *et al.* (2004) first suggested the detection of infected plants at an early stage based on measurement of released volatiles. In the same year, Holopainen (2004) concluded: ‘Non-

destructive metabolic profiling of VOC emissions is a promising tool for quickly detecting the physiological status of crop plants as well as for identifying the initial phase of pathogen and herbivore infections'. To the best of our knowledge Baratto *et al.* (2005) were the first group to report experiments on monitoring plant health status based on measurement of emitted volatile compounds. Their research indicated that a gas sensor is able to detect the onset of plant stress due to insect damage or herbicide spraying.

The objective of this study was to assess the potential of VOC sensing as a non-invasive method for plant diagnosis. To test this premise, we measured the emission of VOCs from tomato plants infected with the fungus *B. cinerea*.

MATERIALS AND METHODS

Plant chamber

The chamber used for the experiments was described in Heiden *et al.* (2003). In brief, a glass chamber with a volume of 1.1 m^3 was mounted in a temperature-controlled housing (Fig. 3.1). This plant chamber was supplied with entries for temperature and light intensity sensors and connected tubings for gas-phase analysis and air supply. Only glass and Teflon was used in construction because these materials are known to reduce adsorption of VOCs on walls of the apparatus (Stewart-Jones and Poppy, 2006). The glass chamber was illuminated with metal halide lamps (Powerstar HQI 400 W/D lamps; Osram, Munich, Germany). To prevent overheating of the plants by infrared radiation, filters (Prinz Optics GmbH, Stromberg, Germany, type IR3) that reflect wavelengths between 750 and 1050 nm were used as heat shields. Digital mass flow controllers were used to control the air flow through the chamber at 50 and 80 L min^{-1}. Incoming air was purified through an adsorptive drying device (Zander Aufbereitungstechnik GmbH & Co. KG, Essen, Germany) and by a palladium catalyst. Humidity was maintained by passing incoming air through a glass vessel containing bidistilled water. The CO_2 concentration in the chamber could be adjusted by adding CO_2 to the chamber inlet. A Teflon fan was used to mix the air in the chamber and to reduce boundary layer resistance at leaf level. A personal computer with a data-acquisition system based on LabVIEW software (National Instruments, Austin, TX, USA) recorded temperature, dew point, and light intensity in the chamber, airflow through the chamber, and the differences in concentration of CO_2 and water vapour between the chamber inlet and outlet.

Teflon sheets were used to separate the aerial part of the plants from the roots and substrate to prevent contamination of the air in the chamber by emissions from the substrate and roots. The plant stems were positioned through holes in these sheets and sealed airtight with Optosil P (Heraeus Kulzer GmbH, Hanau, Germany).

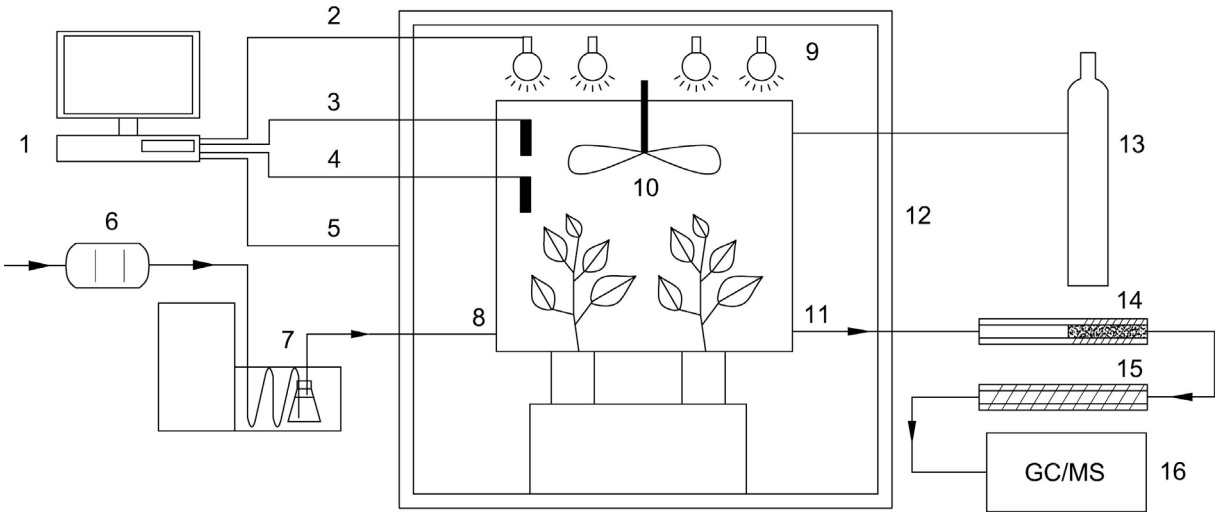

Fig. 3.1 Schematic overview of the experimental set-up: (1) personal computer, (2) light control, (3) light intensity sensor, (4) dew point sensor and (5) temperature sensor, (6) air filters, (7) humidifier, (8) chamber inlet, (9) lights, (10) Teflon fan, (11) chamber outlet, (12) temperature-controlled housing, (13) CO_2 supply, (14) thermal desorption system, (15) cryofocussing device and (16) gas chromatography – mass spectrometry (GC/MS).

Gas chromatography – mass spectrometry

Two different gas chromatography – mass spectrometer systems (GC/MS) were used for the measurements. The first GC/MS system (system A) is described in detail by Heiden *et al.* (1999). This system is based on a HP 5890 Series II gas chromatograph with a quadrupole mass selective detector HP-MSD 5972A. System A was optimised to quantitatively detect volatiles such as products from the lipoxygenase pathway (LOX products) and mono- and sesquiterpenes. The second GC/MS system (system B) is thoroughly described in Folkers (2002). This system is based on a Hewlett Packard HP 6890 gas chromatograph with a quadrupole mass selective detector HP-MSD 5973. System B was optimized to quantitatively

detect volatiles such as methanol, LOX products and monoterpenes. Both systems used thermal adsorption/desorption for pre-concentration (Gerstel online TDS G) connected to a cooled injection system (Gerstel; KAS 3 for system A and KAS 4 for system B) where samples were cryofocused before injection onto the column (BPX-5 column, SGE, 50 m × 0.22 mm × 1 µm for system A; CP Wax 52 CB, Chrompack, 60 m × 0.25 mm × 0.5 µm for system B).

In ten out of twelve experiments, system A was used but in two experiments, system A was not available and therefore system B was used. The results and discussion are focused, therefore, primarily to volatile compounds measurable with both systems, *i.e.* LOX products and monoterpenes. Both systems were calibrated at regular intervals to allow intercomparison of data from the individual systems.

Calibration of both GC/MS systems was performed using a permeation source containing pure chemicals in individual vials in combination with a dynamic dilution system. Details of the calibration procedure for these GC/MS systems were described in Heiden *et al.* (2003). Concentrations of the compounds released from the calibration source were determined from the mass loss rates of the individual compounds and the dilution fluxes. The VOC mixing ratios of this source were in the lower ppb to ppt range. The reproducibility of VOC concentration measurements was in the range of 10 and 15% in the upper ppt level. Depending on the fractionation of the individual VOCs, the detection limit of our system was in the range of 0.02 to 0.05 ng for the monoterpenes (≈1.0–2.4 ppt per 4 L sampling volume) and 0.05–0.95 ng for the LOX products (≈3–6 ppt per 4 L sampling volume). To check for losses of VOCs in the empty chamber, isoprene, *cis*-3-hexenol, *n*-decane, *α*-pinene, *β*-pinene, 3-carene, limonene, methyl salicylate (MeSA) and the sesquiterpene longifolene were added to the inlet of an empty chamber. Concentrations of the individual VOC in the inlet air and outlet air of the empty chamber were measured and found to be equal proving that losses through gas phase reactions and adsorption on chamber walls and tubings were negligible for these VOCs.

LOX products and monoterpenes were identified by comparing the mass spectra with mass spectra libraries *i.e.* Wiley mass spectral library, NIST library and a reference library built by measuring commercially available standards (Fluka and Sigma). Corresponding peak areas

were then used to calculate the concentrations of the individual VOC in the sample. It was expected that volatile emission might be influenced by the biomass of the plants and we used the leaf area of the plants to consider the biomass. Flux densities (Φ_M) for the detected VOCs were calculated as follows:

$$\Phi_M = \frac{F}{A} \cdot (c_o(M) - c_i(M))$$

where F is the air flux through the chamber, A is the one-sided leaf area of the investigated plants, c_o is the VOC concentration measured at the chamber outlet and c_i is the VOC concentration measured at the chamber inlet. M represents the VOC of interest. This flux density is the amount of the respective VOC emitted within a certain period of time normalized to the leaf area. As previous measurements indicated that VOC concentrations of incoming air are below the detection limit of the GC/MS, c_i was set to zero.

Plant material

Seeds of tomato (*Lycopersicon esculentum* Mill.) cv Moneymaker were germinated in a commercial mixture of soil, peat, and compost (Pikiererde, Plantaflor, Vechta, Germany). After 10 days, the seedlings were transplanted into individual containers containing standard substrate (Einheitserde, type ED 73). The plants were germinated and grown in a controlled climate chamber at 20°C and 50% relative humidity (RH). Plants were grown at a photosynthetic photon flux density (PPFD) of 300–400 µmol m^{-2} s^{-1} with a 12-h light/12-h dark day-night photoperiod. Typically, plants were 6–8 weeks of age when used in the experiments. At that age, the individual plants were between 0.6 and 0.8 m high. During the experiments the plants were regularly watered to prevent drought stress. After each experiment, the leaves were cut from the plants to determine total leaf area. This total leaf area was used to calculate gas flux per unit area.

***Botrytis cinerea* inoculum**

Botrytis cinerea strain B0510 was prepared on malt extract agar (CM0059, Oxoid, Bastingstoke, UK) as described by Benito *et al.* (1998). The final spore suspension consisted of 50 ml filter-sterilized water supplemented with 0.6 g potato dextrose medium (Duchefa

Biochemie BV, Haarlem, The Netherlands). The concentration of spores in the suspension was counted using microscopy and adjusted to 1 $\times 10^6$ spores ml^{-1}. Per experiment, leaves of three or four tomato plants were inoculated on the ventral leaf surface using a micro-sprayer. Each plant was sprayed with ~15 ml of the spore suspension. The climate in the chamber was then set to 15°C and a RH exceeding 95%.

Experimental design

In total, 12 independent experiments with a total of 40 plants were conducted. For each experiment, three or four tomato plants were inserted into the plant chamber. The plants were allowed to adapt to the climate in the plant chamber before spraying. Then, in six out of the 12 experiments the plants were sprayed with a suspension of potato dextrose medium containing *B. cinerea* spores (three to four plants per replicate). In the six control experiments, plants were sprayed with the identical suspension of potato dextrose medium lacking *B. cinerea* spores (three plants per replicate). After application of the suspension, the lights were turned off for 16 h, the RH was increased to above 95%, and chamber temperature was 15°C. Such a high RH is known to favour penetration of *B. cinerea* into tomato leaves. After 16 h in darkness, the lights were manually switched on for 8 h followed by 12-h/12-h light/dark regime. The four different phases used in all experiments as listed in Table 3.1.

Table 3.1 Definition of phases for plants sprayed with or without *Botrytis cinerea* spores. Photosynthetic photon flux density (PPFD) in each light phase was 480 μmol m^{-2} s^{-1}. Temperature during light phases was 18°C, and during darkness was 15°C.

Phase	Interval (± 1 h)	Illumination
1	0 – 16 h after spraying	Dark
2	16 – 24 h after spraying	Light
3	24 – 36 h after spraying	Dark
4	36 – 48 h after spraying	Light

The temperature during the light and dark phases was maintained at 18°C/15°C, respectively. PPFD during all light phases was 480 μmol m^{-2} s^{-1}. CO_2 concentration was maintained at approximately 350 ppm to mimic as much as possible natural environmental conditions. VOC

emissions depend on temperature and light (Schuh *et al.*, 1997). Therefore control of these environmental conditions is important to obtain reliable quantitative results in VOC analyses Helsper *et al.* (2002). To allow reliable interpretation of volatile emissions from plants the variations in temperature and light were strictly maintained for all investigated plants.

Volatile organic compounds were measured at hourly intervals starting two h after spraying. This procedure was followed because the high RH directly after spraying can cause severe technical problems to the GC/MS equipment. Development of necrotic spots on plants during the experiments was visually monitored. Unfortunately, the thick glass walls of the chamber prevented accurate monitoring with a camera system. After the experiments, leaves were removed from the plants and total leaf area was determined for calculating the flux density, as described earlier. Digital images of detached leaves were recorded for leaf area assessment.

RESULTS AND DISCUSSION

Visual observation of infection symptoms

There was a variation in the number and expansion rate of lesions between the replicates. In four experiments, the first symptoms were small necrotic spots on some of the leaves approximately 24 h after inoculation. These spots remained small and restricted throughout the whole experiment, *i.e.* for two days after inoculation. Hereafter, these four replicates will be referred to as “mild infection”. In two experiments, the lesions expanded rapidly, resulting in large unrestricted dry regions on leaves. These two replicates will hereafter be referred to as ‘severe infection’. Such large differences in leaf lesion expansion rates between different inoculation experiments are common and have been observed on tomato plants have been infected with *B. cinerea* (ten Have *et al.*, 2007). Typical examples of mild and severe infection symptoms are provided in Fig. 3.2.

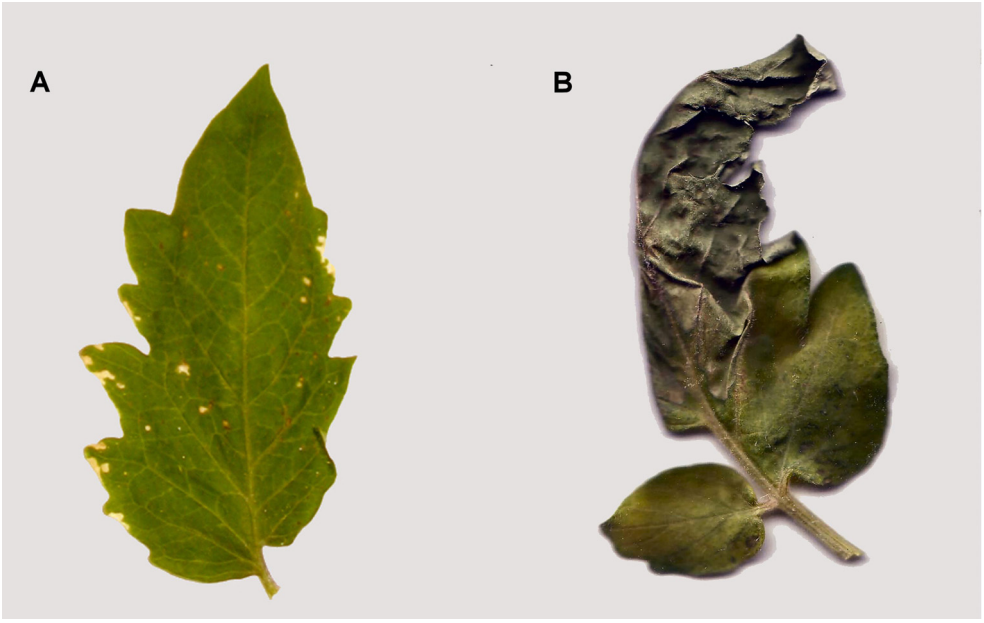

Fig. 3.2 Visible symptoms as observed after the experiments. A: Mild infection symptoms characterised by the presence of small necrotic spots. B: Severe infection symptoms, in which the lesions expanded and covered almost 50 % of many leaves.

Emissions of LOX products

Volatile products from the lipoxygenase pathway (LOX products) are often called green leaf volatiles and can account for > 50% of emissions from damaged plant parts (Holopainen, 2004). Up to nine different LOX products were identified in the volatile emission from tomato plants after inoculation with the fungus *B. cinerea*. The most dominant LOX products were the C_6-compounds *cis*-3-hexenal, *cis*-3-hexenol, and *trans*-2-hexenal; the C_5-compound 1-penten-3-ol; and the C_8-compound *cis*-3-hexenyl-acetate. In all experiments resulting in LOX product emission, the sum of these five compounds contributed about 90% to total LOX product emission (data not shown). Therefore, we consider these compounds as the sum of all LOX products, hereafter referred to as Σ LOX. As emissions were analysed every hour, it was possible to study the time courses of Σ LOX. Fig. 3.3 shows typical examples for time courses of Σ LOX products from control plants and plants developing mild and severe infections.

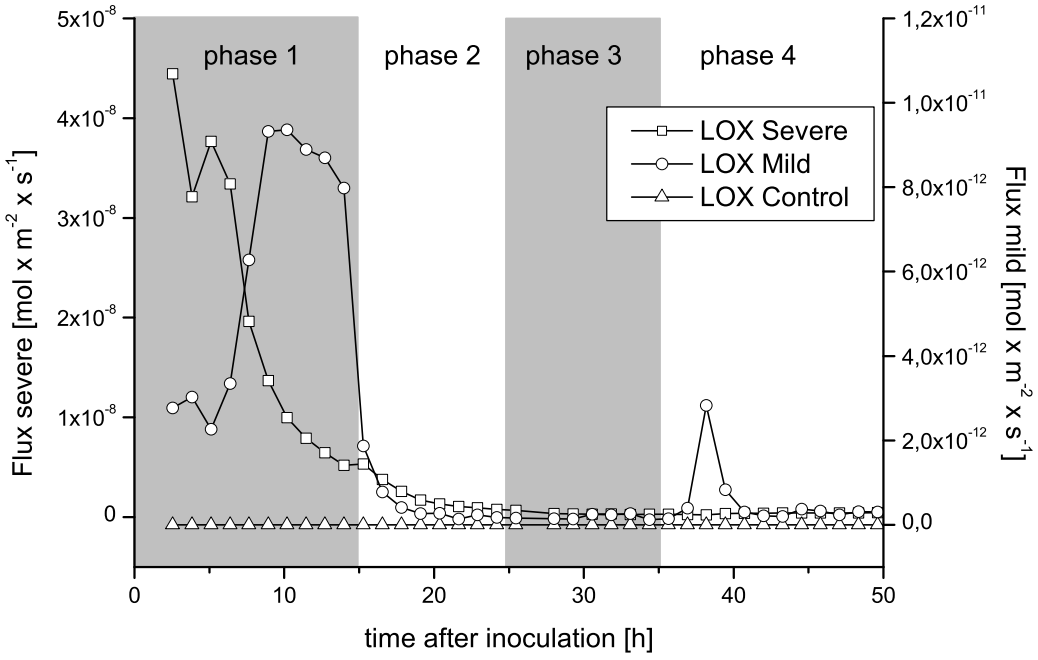

Fig. 3.3 Typical time courses of the combined emission (Σ LOX) of the five most dominant volatiles from the lipoxygenase pathway (*cis*-3-hexenal, *cis*-3-hexenol, *trans*-2-hexenal, *cis*-3-hexenyl-acetate, 1-penten-3-ol) by tomato plants inoculated with *Botrytis cinerea* and by control plants. Squares (left) refer to an experiment in which plants developed a severe infection. Open circles (right) refer to an experiment in which plants developed a mild infection. Triangles refer to a control experiment. Shaded areas represent dark phases. GC/MS measurements started at 2 h after spraying in order to prevent technical problems with the GC/MS equipment due to the high humidity directly after spraying.

In all experiments in which plants were inoculated with *B. cinerea*, emissions of LOX products were found. These emissions were not found from control plants sprayed with a suspension without fungal spores. The emissions of LOX products reached a maxima during phase 1 *i.e.* within the first 16 h after inoculation. Thereafter, the emissions steadily decayed on time scales of hours in three cases. However, in three other cases (see Fig. 3.3) small additional pulses of LOX product emissions were observed 16-40 after inoculation. These latter pulses were lower than those found during phase 1 and lasted for shorter times.

The delay between inoculation and maximum LOX product emissions of the first large pulses differed in each experiment. In particular, large differences were observed between mild and severe infection. For mild infections, the maximum emission appeared approximately 10 h after inoculation whereas for the severe infections the maximum emission seemed to have appeared already before the start of measurements.

Concurrent with the earlier emission of LOX products from tomato plants showing severe symptoms, the absolute amount of emitted LOX products was also about three orders of magnitude higher compared to plants showing mild symptoms. Table 3.2 lists the minima and maxima of LOX product emissions for the different experiments and different phases.

Table 3.2 Range of emission of Σ LOX products (mol m^{-2} s^{-1}).

	***Botrytis cinerea* severe (*n*=2)**		***Botrytis cinerea* mild (*n*=4)**		**Control (*n*=6)**	
	Min	Max	Min	Max	Min	Max
Phase 1	5.19E-9	4.44E-8	2.2E-16*	3.59E-11**	n.d.	n.d.
Phase 2	6.83E-10	2.65E-8	8.15E-16	1.98E-11	n.d.	n.d.
Phase 3	2.28E-10	1.27E-9	1.1E-16*	8.92E-12	n.d.	n.d.
Phase 4	3.93E-10	9.93E-10	2.1E-16*	7.60E-12	n.d.	n.d.

* data near to the detection limit; ** *n*=3

n.d. = not detected, *i.e.* < 5E-17 which is the detection limit for the conditions used here.

Grades of infection were classified as mild or severe, although such a qualitative classification is arbitrary and no clear limits for such classes. The severity of infection in the case of tomato plants infected with *B. cinerea* might span the whole range of data listed in Table 3.2 or may be even higher. Despite this arbitrary classification of infection grade, we found severe symptoms of injury accompanied by larger emission of LOX products. Similarly, mild symptoms were accompanied by lower emissions of LOX products. We attribute this relation between the amount of LOX product emissions and the severity of infection to the degree of leaf membrane degradation (see Fig. 3.2). Such relations between the emissions of LOX products and the degree of membrane degradation of plant tissue are consistent with those of Fall *et al.* (1999) and Beauchamp *et al.* (2005). Fall *et al.* (1999) reported an increase in LOX product emissions from aspen leaves that was proportional to the extent of wounding and thus to the degree of membrane degradation. Beauchamp *et al.* (2005) reported a close relationship between the amount of emitted LOX products and the degree of stress due to ozone exposure of *Nicotiana tabacu*. where increased ozone levels results in the formation of necrotic lesions (Iriti *et al.*, 2006) and hence membrane degradation. Similarly, in the case of *B. cinerea* infection of tomato plants, the amount of LOX product emitted as well as well as the timing of emission, depends on membrane damage caused by the infection.

The timing of the emissions of individual LOX products differed slightly from that of the sum of LOX products (not shown). This behaviour was also observed for tobacco plants infected with *Pseudomonas syringae* (Heiden *et al.*, 2003). These authors reported time shifts between the pulses of individual volatile LOX products up to 2 h that could be attributed to the consecutive production of LOX products. Such time shifts are caused by the parallelism of production and emission and hint at processes after the production of the first volatile C_6 LOX product, *cis*-3-hexenal (*e.g.* Croft *et al.*, 1993). Because we wanted to examine the influence of *B. cinerea* attack on induction of the enzyme sequence producing all LOX products, we summed the emission rates of the main LOX products. This procedure cancels out the impact of conversion of one VOC into another and provides the temporal course of the steps until the production of *cis*-3-hexenol. It is likely that the time course of this sum of LOX product emissions basically reflects the time course of membrane degradation as observed in *Phragmites australis* exposed to high temperature (Loreto *et al.*, 2006).

Emissions of monoterpenes

In addition to the emissions of LOX products, up to 16 different monoterpenes were identified in the volatile emissions from tomato plants. The most dominant were of β-phellandrene, 2-carene, limonene, α-phellandrene, and α-pinene. These five monoterpenes contributed to more than 95% of the volatile monoterpene emission in all experiments (data not shown). Therefore we considered these compounds as the sum of all monoterpene emissions, hereafter referred to as Σ monoterpenes. Fig. 3.4 shows an example of the time course of Σ monoterpene emission after mild and severe infection as well as for control plants.

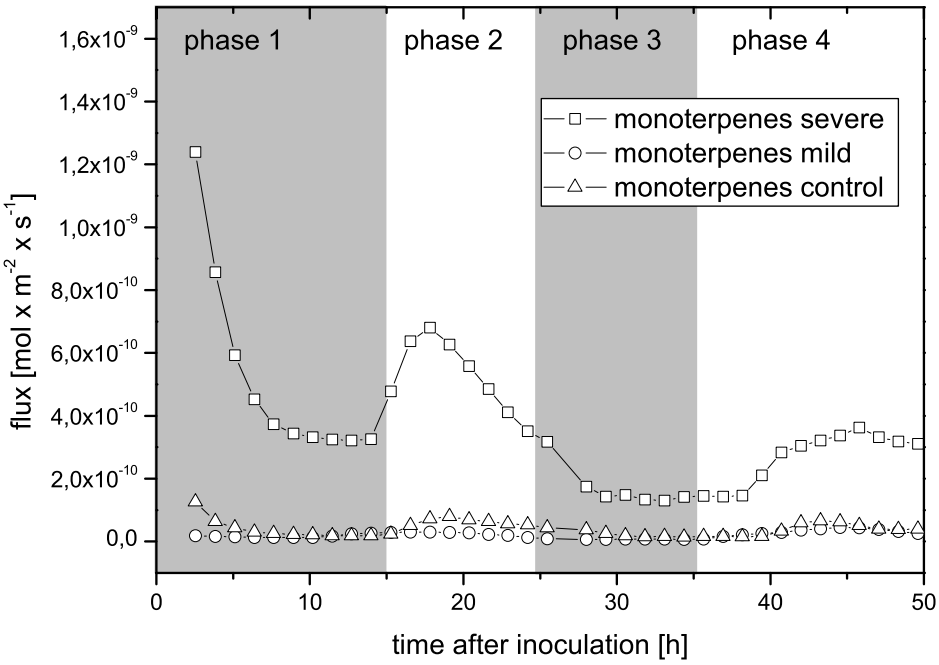

Fig. 3.4 Time course of combined emission of monoterpenes (Σ monoterpenes) for the five most dominant monoterpenes (*β*-phellandrene, 2-carene, limonene, *α*-phellandrene, *α*-pinene) by tomato plants inoculated with *Botrytis cinerea* and by control plants. Data are shown from three experiments: one experiment resulted in severe infection (squares), one experiment resulted in mild infection (circles) and one is from control plants (triangles). Shaded areas represent dark phases. GC/MS measurements started at 2 h after spraying in order to prevent technical problems with the GC/MS equipment due to high humidity directly after spraying.

After starting the measurements, large monoterpene emissions were observed in case of severe *B. cinerea* infection. These emissions decreased over a time scale of hours and varied according to the day/night rhythm thereafter. Light-dependent emissions of monoterpenes by plants has been frequently described (Tarvainen *et al.*, 2005), as has temperature-dependent emissions (Schuh *et al.*, 1997). Both factors may simultaneously control the emission of VOCs from plants. However, for tomato, we believe that the day/night rhythm of monoterpene emissions is mainly temperature controlled. Such temperature dependent emission of monoterpenes has been found in plant species that store volatile organic compounds in pools *e.g.* pine trees, which store monoterpenes in their needles (*e.g.* Loreto *et al.*, 2000) and tomato plants which store monoterpenes in trichomes (van Schie *et al.*, 2007). These studies support our assumption that temperature is the main factor controlling the

day/night rhythm of monoterpene emission in tomato. One method to elucidate the role of volatile storage pools with respect to pathogen-induced plant emissions is labelling of inoculated plants with ^{13}C-CO_2 in parallel with GC/MS analysis of volatile compounds released (for details, see Paré and Tumlinson, 1997).

Table 3.3 lists the ranges of Σ monoterpenes (the five monoterpenes listed above) measured for 2 days after spraying. The baseline emissions of monoterpenes due to diffusion out of trichomes have been described for tomato and other crops (Bäck *et al.*, 2005, Loughrin *et al.*, 1994, Maes and Debergh, 2003). It is possible that the release of monoterpene emissions may depend on the amount of trichomes on a plant. In such case the emission strength may vary from individual to individual, which explains the variability of monoterpene emissions from control plants.

Table 3.3 Range of emissions of Σ monoterpenes (mol m^{-2} s^{-1}).

	***Botrytis cinerea* severe (*n*=2)**		***Botrytis cinerea* mild (*n*=4)**		**Control (*n*=6)**	
	Min	Max	Min	Max	Min	Max
Phase 1	3.2E-10	8.6E-10	2.8E-13*	5.4E-11*	2.3E-12	9.2E-12
Phase 2	3.2E-10	1.4E-9	3.0E-13	2.3E-11	2.1E-12	2.2E-11
Phase 3	1.3E-10	5.1E-9	1.1E-13	8.5E-11	1.7E-12	5.3E-12
Phase 4	3.0E-10	5.1E-10	2.3E-13	3.4E-11	4.7E-12	1.71E-11

* *n*=3.

To further evaluate the emission of monoterpenes, we examined the pattern of this class of compounds. Interestingly, the emission patterns of monoterpenes were independent of the time after inoculation; there was an almost perfect linear correlation (Pearson $r > 0.9$) between the emissions of individual monoterpenes. Fig. 3.5 shows the correlation of emission rates of limonene, α-phellandrene, 2-carene and α-pinene *versus* β-phellandrene for an experiment that resulted in severe infection.

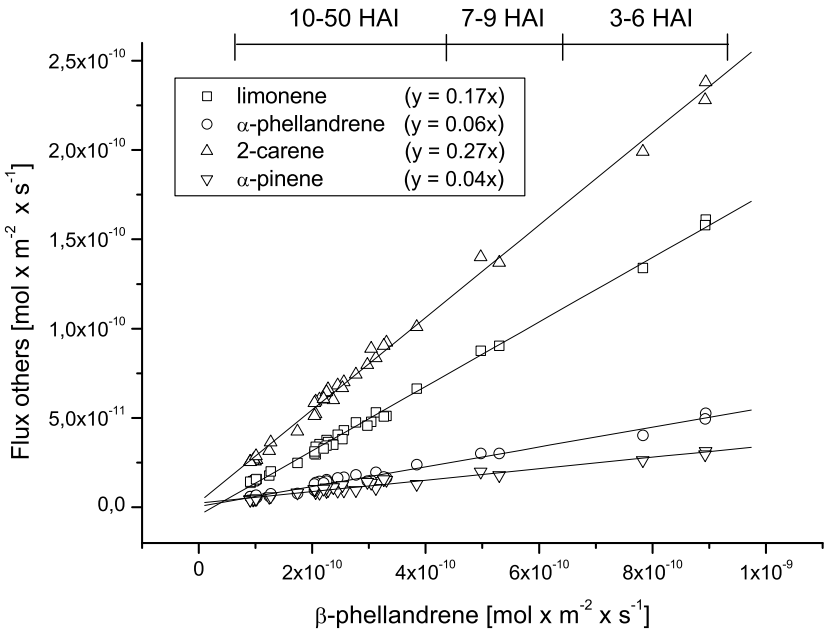

Fig. 3.5 Correlation between emission rates of limonene, α-phellandrene, 2-carene and α-pinene *versus* β-phellandrene. Data are shown from an experiment that resulted in severe infection. HAI = hours after inoculation.

The monoterpene emission pattern was constant over time, independent of the treatment and independent of the extent of infection. Control plants as well as inoculated plants showed this pattern in monoterpenes, suggesting the same mechanism controls the emission of all of these compounds. This mechanism is probably related to diffusion rates from trichomes.

In plants with severe symptoms, there was an increase in monoterpene emissions (Table 3.3). A severe *B. cinerea* infection resulted in large unrestricted dry regions on leaves (Fig. 3.2B) with destruction of the trichomes in such regions. These tomato leaf glandular trichomes contain a rich spectrum of monoterpenes (Colby *et al.*, 1998). It is therefore likely that damage of trichomes as a result of severe *B. cinerea* infection caused the observed increase in monoterpene emissions. In case of mild symptoms, no impact of *B. cinerea* infection on the monoterpene emissions was detectable (Table 3.3). In these cases inoculation with *B. cinerea* resulted in small restricted spots on the leaves (Fig. 3.2A) suggesting that few trichomes were damaged, which resulted in levels of monoterpene emissions that are indistinguishable from the baseline emissions of control plants.

If baseline monoterpene emissions are due to diffusion out of trichomes and increased monoterpene emissions are due to mechanical damage to trichomes, the monoterpene pattern should stay constant and should be independent of the type of stress. This was observed in the present experiments, suggesting that the main part of the strong monoterpene emissions from tomato with severe symptoms is due damaged trichomes. Several studies have showed increased monoterpene emissions upon damage to tomato plants (Dicke *et al.*, 1998, Farag and Paré, 2002, Kant *et al.*, 2004, Maes, 2002, Sanchez-Hernández *et al.*, 2006, Thaler *et al.*, 2002, Wei *et al.*, 2007) as a result of herbivore infestation. Nevertheless, the increase in monoterpene emission from tomato upon herbivory also probably resulted from damaged trichomes since herbivore species have the ability to damage trichomes (*e.g.* Gibson, 1971). If our assumption of damaged trichomes being the main reason for the increased monoterpene emissions after *B. cinerea* infestation is correct, it follows that the monoterpene emission pattern after herbivore attack or *B. cinerea* infestation would be the same. Monoterpene emissions from tomato therefore cannot be used to identify the stressor leading to the damage.

Many plants respond systemically to stressors and then emit VOCs not only at the site of damage, but also from remote, undamaged tissues (*e.g.* Tingey *et al.*, 1991, Turlings and Tumlinson, 1992). In addition to trichome damage, a second possibility for an increase in monoterpene emissions may be such a systemic response following a *de novo* synthesis of monoterpenes in the whole plant. Farag & Paré (2002) found that tomato plants treated with chemicals release *trans*-2-hexenal, a LOX product emitted by tomato after *B. cinerea* infestation; therefore an induced systemic response cannot be strictly excluded. Furthermore, Farag & Paré (2002) also reported that the local response *i.e.* a mechanical damage of trichomes might lead to a stronger increase in these monoterpene emissions. This is consistent with our assumption that trichome damage is the main reason for increased monoterpene emissions. However, independent of whether the increase in monoterpene emissions was due to a systemic or a local response, the emission pattern did not change. Hence, systemic induced emission would be indistinguishable from local damage. Without further information the exact mechanism of increased monoterpene emissions can therefore not be identified.

Systemic wound responses appear to be ubiquitous in plant-insect interactions (Heil and Ton, 2008). For example, an increase in the systemically emitted compounds methyl salicylate and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) was reported upon herbivore

infestation of cotton (Röse *et al.*, 1996). Results from our study indicated a similar increase in emissions of methyl salicylate and TMTT after infection with *B. cinerea*. According to van Schie *et al.* (2007), these compounds are not stored in trichomes of tomato; therefore, they are likely to be systemically induced upon infection with *B. cinerea*. However, also these emissions are probably not specific to any type of stress since stress due to enclosure of tomato plants –a prerequisite for analysing plant emission- also led to increased emissions of methyl salicylate and TMTT (Kant *et al.*, 2004).

CONCLUSION

The objective of this study was to assess the potential for VOC sensing as an early and non-invasive method for diagnosis of tomato plant infection by *B. cinerea*. This approach seems applicable; infection of tomato plants with *B. cinerea* resulted in rapid changes in VOC emissions. Among the volatile compounds measured, products from the lipoxygenase pathway (LOX products) were the strongest indicator of the stress response. Besides emissions of LOX products, there were also increases in monoterpene emissions . However, neither emissions of LOX products nor increased monoterpene emissions can be used to identify the stressor. LOX products emissions basically reflect membrane injury and therefore emission of LOX products can only be used to identify plant tissue damage in an unspecific way. Also monoterpene emissions seem to be independent of the origin of the plant tissue damage. In order to draw conclusions about the elicitor of plant tissue damage it is necessary to find volatile emissions that are unique for an elicitor.

Among the main compounds emitted from tomato infected with *B. cinerea*, we found no such unique emission pattern. Nevertheless, unspecific emissions can be used for early stress detection. For identification of the stressor itself, additional information is necessary from several additional criteria. For example, outbreaks often involve a single disease with specific factors for the outbreak, such as high humidity. Such criteria should thus be taken in account when evaluating VOC emissions from plants. For practical purposes, it is advisable that most common diseases known to occur in greenhouses should be studied. Furthermore, scale-up experiments are required to obtain more information regarding the applicability of VOC sensing as a non-invasive method for diagnosis of disease prevalence in greenhouses.

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CHAPTER 4

The effect of trichome damage and cell membrane damage on the concentration of volatiles in a small-scale greenhouse

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INTRODUCTION

Pathogen attack and herbivore infestation have a major impact on plant health and therefore cause considerable crop yield losses in greenhouse cultivation (de Clercq, 2001). A monitoring system to reveal plant health problems at an early stage would facilitate immediate interventions and possibly diminish adverse effects. Imaging techniques to monitor plant health have recently been reported (Boissard *et al.*, 2008, Chaerle *et al.*, 2006). These imaging techniques provide an important contribution to current research related to early and non-invasive plant diagnosis (see Liew *et al.*, 2008). Critical disadvantages of imaging techniques are that they cannot provide information on inner canopy layers and can detect disease problems only after visual symptoms have developed. A more adequate method for monitoring plant health should provide information on both inner and outer canopy layers and should warn for problems at an early stage.

An attractive method to provide plant health information is the analysis of volatile organic compounds (VOCs) in greenhouse atmosphere. This new idea originates from the numerous laboratory experiments, which revealed that stresses of plants change the volatile blend released by the plant. For example, pathogen infection of various plants has elicited the emission of several VOCs (Deng *et al.*, 2004b, Shulaev *et al.*, 1997, Vuorinen *et al.*, 2007). Also herbivore infestation resulted in the release of many different types of VOCs (Röse and Tumlinson, 2005, Takabayashi *et al.*, 1994).

In the present study, three mechanisms were regarded as important with respect to stress-induced emissions from plants. The first factor is the damage of storage pools in plants that contain liquids that are readily emitted after damage. An example of this is the emission of monoterpenes as a result of damage to glandular trichomes due to the necrotic pathogen *Botrytis cinerea* (Jansen *et al.*, 2009c). The second factor is cell membrane damage. When cell membranes are damaged, several chemical substances are emitted due to the breakdown of membrane lipids (Paré and Tumlinson, 1999). These emissions consist of a blend of saturated and unsaturated six-carbon alcohols, aldehydes, and esters derived from the lipoxygenase pathway and thus commonly referred to as LOX products (Beauchamp *et al.*, 2005). The third factor concerns the systemic response in which plants emit VOCs not only at the site of damage, but also at undamaged leaves (Turlings and Tumlinson, 1992).

In this study, tomato plants were used to study the feasibility of monitoring plant health through analysis of VOCs in greenhouse atmosphere. Several plant health issues are relevant in tomato-producing greenhouses. For example, herbivorous spider mites (*Tetranychus urticae*) form a serious pest in tomato crops (van Leeuwen *et al.*, 2005). Also grey mould, caused by the necrotrophic pathogen *B. cinerea*, is a well-known cause for considerable damage in tomato production (Elad and Stewart, 2004). Although we aimed to investigate whether plant health monitoring based on plant VOCs is feasible, we decided not to use herbivores or pathogens because these types of stressors are difficult to control and might fully destroy the crop within a short period not allowing any replicate measurements. To simulate pathogen attack and herbivore infestation, we repeatedly applied two types of controlled damage to the crop: (1) damage as a result of stroking the stems, and (2) damage due to removing side shoots.

Stems of the plants were stroked with the intention to damage the trichomes located on the stem. To use simulated pathogen attack and herbivore infestation by trichome damage is relevant because also pathogens and herbivore species have the ability to damage trichomes (*e.g.* Gibson, 1971). Therefore, the first objective of this research was to study the effect of trichome damage on plant-emitted VOCs in greenhouse atmosphere.

Removal of side shoots was applied with the intention to inflict cell-membrane damage. To use simulated pathogen attack and herbivore infestation by cell membrane damage was supposed to be adequate because cell membranes are also damaged as a consequence of pathogen infection and herbivore feeding (Levin, 1976). Therefore, the second objective of this research was to study the effect of cell membrane damage on plant-emitted VOCs in greenhouse atmosphere.

During the commercial cultivation of tomato, the mature fruits are often harvested. Likely, this treatment also results in the modification of plant emission and thus the concentration of plant-emitted VOCs in the greenhouse air. Because fruit picking occurs frequently in commercial greenhouses, this treatment might obstruct the interpretation of pathogen- and herbivore-induced volatiles during the monitoring of plant health status. Therefore, the third objective of this research was to study the effect of fruit picking on the concentration of plant-

emitted VOCs in greenhouse atmosphere. The findings presented in this paper focus on the feasibility of monitoring plant health through the analysis of VOCs in greenhouse air, but we also note the atmospheric significance of regular crop activities such as harvest.

MATERIALS AND METHODS

Plant material

Seeds of tomato plants (*Lycopersicon esculentum* Mill.) of the cultivar Moneymaker were germinated in a standard greenhouse at 20°C and 50% relative humidity. When plants were about seven weeks old, 60 plants were transferred to a small experimental greenhouse. At that age the individual plants were about 80 cm high. Plants were placed on rockwool slabs and irrigated with standard nutrient solution with the aid of a trickle irrigation system. Experiments were carried out until the plants had nearly reached the lamps located at 2.5 m above ground level (usually about 12 weeks after planting).

Experiments

Four tomato crops were grown over a period of 1 year. Three of these were mainly used for the analysis of greenhouse air before and after stroking of the plants. One was mainly used to study the effect of shoot removal. The first study was from February to March 2007, the second from April to May 2007, the third from June to July 2007, and the fourth from November 2007 to January 2008. During the time period of six weeks, on Wednesday, shoots were removed and plants stems were tied up to hanging wires for support. On Thursday, trichomes were mechanically damaged on a weekly interval by stroking the full length of the stem of each individual plant using a stainless steel bar. Several times, the tomato fruits were picked at the red-ripe stage. Trichome damage, shoot removal, and fruit picking were performed at a fixed starting time point *i.e.* 13.00 h.

Greenhouse equipment and climate control

The experimental greenhouse used for the experiments has been described by Körner *et al.* (2007). In short, the floor area of the greenhouse was 44 m^2 and the total volume including the basement underneath was 270 m^3. Using tracer gas measurements, the air exchange was determined to be 2 mol air sec^{-1} (data not shown). A fan located in the basement was used to

maintain a constant internal air circulation of 20×10^3 m^3 h^{-1}. Electrical heating and direct mechanical cooling situated in the basement controlled temperature and humidity. A day length of 16 h (6:00 h–22:00 h) was maintained with 30 luminaries (SGR; Philips, Somerset, NJ, USA) equipped with one 200 W lamp each (SON-T Agro; Philips). During this period, light was switched on when outside global radiation dropped below 150 W m^{-2} and switched off again when the radiation outside increased above 250 W m^{-2}. The temperature was set at 22.0/16.0 ±1.0°C day/night. The relative humidity inside the greenhouse was maintained at about 70/90 ±5% day/night. Temperature and relative humidity of the air in the greenhouse were measured with dry and wet bulb platinum-resistance temperature sensors. A third sensor measured temperature and relative humidity for climate control purposes. Pure CO_2 was injected into the greenhouse proportionally to the difference between measured CO_2 concentration and the CO_2 set point concentration (420 ppm) using an infrared gas analyser (URAS 3G; Hartmann & Braun, Frankfurt, Germany) and a mass-flow controller (5850E; Brooks, Veenendaal, The Netherlands).

Air sampling in the greenhouse

Continuous flow pumps were used to purge 18 L of air from the greenhouse through stainless steel cartridges (Markes International Ltd, Lantrisant, UK) packed with 200 mg of Tenax-TA 20/35 (Grace-Alltech, Breda, The Netherlands). Air was drawn through these cartridges at 300 mL min^{-1} for 60 min. Air samples were collected before and directly after stroking the plants as well as before and directly after shoot removal. In the initial experiments, the greenhouse atmosphere was sampled at three locations within the greenhouse to provide insight into the spatial distribution of VOCs inside the greenhouse. These three sampling points were located in the left-rear, centre, and right-front location of the greenhouse at a height of 1 m. Results indicated that the concentration differences between the three locations were small (approximately 10%). The similarity in concentration was expected because of the large internal air circulation. Therefore, samples obtained in the centre were used in the remainder of this study. After sampling, the cartridges were immediately capped and transferred to the lab for analysis. In between the replicates, we performed empty greenhouse tests which showed no indication of VOCs emitted from greenhouse materials or VOC-transfer from outside to inside of the greenhouse that could be misleadingly interpreted as plant emissions.

Volatile analysis

The analysis of volatile compounds using gas chromatography coupled to mass spectrometry (GC-MS) has been described before (Jansen *et al.*, 2008). Before analysis, the cartridges were dry-purged with helium at ambient temperature with a flow of 100 mL min^{-1} for 10 min to remove water. Analytes were desorbed from the cartridges using thermal desorption at 250°C for 5 min under a flow of 30 mL min^{-1} of helium, and subsequently concentrated in an electronically-cooled focusing trap at -5°C (UltrA-TD™ and Unity™; Markes International Ltd.). Analytes were then transferred to the column by heating the cold trap to 250°C at approximately 40°C sec^{-1}. The column (Rtx-5 MS, 30 m × 0.25 mm internal diameter × 1 μm film thickness; Restek, Bellefonte, PA, USA) was held at the initial temperature of 40°C for 3.5 min followed by a linear gradient of 10°C min^{-1} to 280°C and a hold of 2.5 min resulting in an overall runtime of almost 33 min. The column flow was approximately 1 mL min^{-1}. A multitube conditioning unit was used for cleaning the cartridges in between the measurements at 310°C for 40 minutes under a flow of 50 mL min^{-1} of helium. To prevent overloading of the analytical system, most samples were split prior to injection. Air samples obtained when plants were relatively small were analysed in splitless mode while samples obtained in case of large plants were analysed at split inlet modes between 1:6 and 1:24. To correct for the split levels used, data were normalized to naphthalene. Naphthalene was selected for normalization because this compound is not released from plants and was always present in almost constant concentration inside the greenhouse (Jansen *et al.*, 2008). The response of the GC-MS to the standard naphthalene was also used to correct for variability in GC-MS sensitivity (*e.g.* because of contamination of the ion source and after cleaning the ion source).

For quantification purposes, an experimental set-up to provide well defined concentrations of relevant VOCs was used (Heiden *et al.*, 2003). The set-up consisted of several 1 L double walled glass containers that were temperature controlled at 30°C. Each of these containers contained one 4 mL amber glass vial closed by a Teflon plate. These vials contained GC-grade standards of (*Z*)-3-hexenol, *α*-pinene, *β*-caryophyllene and methyl salicylate (Fluka, Milwaukee, WI, USA). Standards with high vapour pressure permeated through the Teflon plate. In case of VOCs with low vapour pressure small holes were punched in the Teflon plates to allow diffusion of the VOC through the small holes. After inserting the vials in the individual containers, the containers were flushed with nitrogen (0.5 to 2 L min^{-1}, purity > 99.99%). At the outlet of the container, a T-piece allowed to split the nitrogen flow containing

the corresponding VOC. About 95% of the flow was discarded and about 5% of the nitrogen flow was mixed with clean air (10 to 30 L min^{-1}). Similar cartridges as used for greenhouse sampling were used for taking samples from this air stream. To calculate evaporation rates, the vials containing the individual VOCs were regularly weighed in time intervals of about two weeks. To calculate the dilution, also the air streams of the splits and the calibration air flow were measured regularly. In a preliminary experiment, two identical cartridges were put in series to test the breakthrough volume, which represents the gas volume above which a given compound is no longer totally trapped. No compounds were trapped in the second cartridge when the sampling volume was 4 L, indicating that the sampling procedure can be used for quantification of the VOCs.

Immediately after analysing the cartridges containing the standards, a sample collected in the greenhouse was analysed to provide an external standard (naphthalene). The response to naphthalene was used to calculate the absolute concentration of compounds in the greenhouse by assuming the responses to be the same as the response of α-pinene for the C_{10}-monoterpenes, methyl salicylate for the phenolics, and β-caryophyllene for the C_{15}-sesquiterpenes and the C_{16}-homoterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

RESULTS

The effect of stroking the plants on VOCs in the greenhouse atmosphere

Chromatographic profiles obtained from the analysis of VOCs in the greenhouse atmosphere contained several peaks that represented compounds from the analytical system itself such as siloxanes, and compounds commonly found in the Earth's atmosphere such as toluene and benzene (Warneke *et al.*, 2001). Besides these signals, the profiles consisted of peaks representing the chemical substances emitted by the plants. Using specific mass-to-charge ratios (*m*/*z*), we could filter out signals representing plant-emitted VOCs such as terpenes and C_6-alcohols; two classes of hydrocarbons generated by plants (Gershenzon and Dudareva, 2007, Hatanaka, 1993, Kesselmeier and Staudt, 1999). A typical chromatographic profile and the effect of filtering out some plant-generated terpenes are presented in Fig. 4.1.

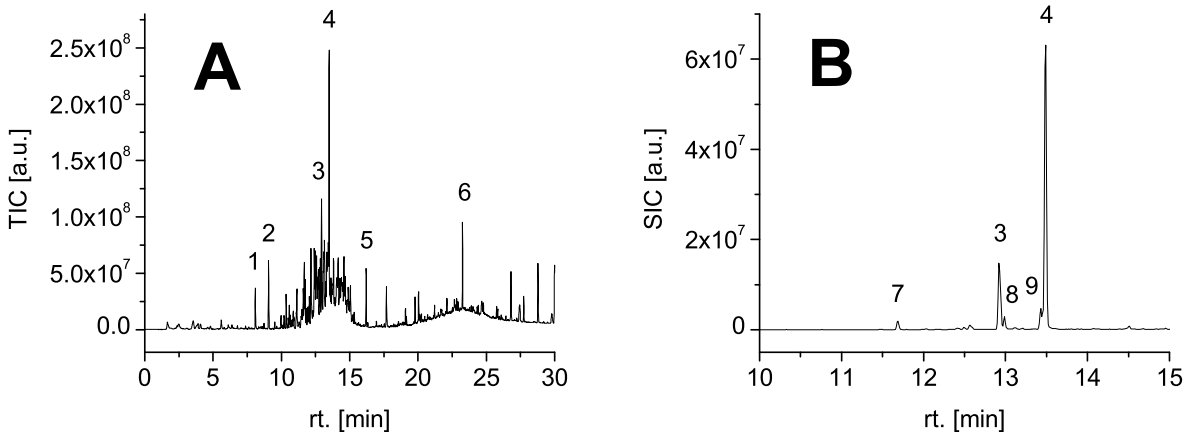

Fig. 4.1 Typical chromatographic profiles obtained from analysing volatile organic compounds in the greenhouse atmosphere. The sample was obtained before stroking when plants were nearly eight weeks old. A) Total ion current (TIC) chromatogram; B) selective ion current (SIC) chromatogram for a selected part of the chromatographic profile using *m*/*z* 93 as representative for monoterpenes. 1 = toluene, 2 = siloxane, 3 = 2-carene, 4 = β-phellandrene, 5 = decanal, 6 = unidentified impurity, 7 = α-pinene, 8 = α-phellandrene, 9 = limonene.

Based on the mass spectra and retention times we could identify up to 17 plant-emitted VOCs in the greenhouse atmosphere before stroking of the plants (Table 4.1). All these compounds were previously described after lab studies in which volatiles emitted from tomato plants were

studied (Deng *et al.*, 2004b, Dicke *et al.*, 1998, Maes and Debergh, 2003, Sanchez-Hernández *et al.*, 2006, Vercammen *et al.*, 2001, Wei *et al.*, 2007).

Table 4.1 Plant-emitted volatile organic compounds detected in the greenhouse before stroking of the plants.*

Compounds	**Formula**	**MW [*m*/*z*]**	**BP [*m*/*z*]**	**Rt [min]**
MONOTERPENES				
α-terpinene	$C_{10}H_{16}$	136	121	13.59
β-phellandrene	$C_{10}H_{16}$	136	93	13.89
α-phellandrene	$C_{10}H_{16}$	136	93	13.38
α-pinene	$C_{10}H_{16}$	136	93	12.07
limonene	$C_{10}H_{16}$	136	68	13.84
2-carene	$C_{10}H_{16}$	136	93	13.32
β-pinene	$C_{10}H_{16}$	136	93	12.96
(*E*)-*β*-ocimene	$C_{10}H_{16}$	136	93	13.99
γ-terpinene	$C_{10}H_{16}$	136	93	14.32
SESQUITERPENES				
β-caryophyllene	$C_{15}H_{24}$	204	41	20.20
δ-elemene	$C_{15}H_{24}$	204	121	18.83
α-copaene	$C_{15}H_{24}$	204	161	19.48
α-humulene	$C_{15}H_{24}$	204	93	20.66
PHENOLICS				
tert-butylphenol	$C_{10}H_{14}O$	134	119	12.79
methyl salicylate	$C_8H_8O_3$	152	120	16.35
p-cymene	$C_{10}H_{14}$	134	119	13.73
OTHER				
(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	$C_{16}H_{26}$	218	69	21.40

BP, base peak; *m*/*z*, mass-to-charge ratio; MW, molecular weight; Rt, retention time.

* Formula, MW, BP, Rt and chemical classes are given for these compounds.

The five most dominant plant compounds in greenhouse atmosphere were the C_{10}-monoterpenes β-phellandrene, 2-carene, limonene, α-phellandrene and α-pinene. The dominance of these five compounds was independent of the age of the plants. Before stroking, concentrations of these five VOCs in greenhouse atmosphere were between 7 pptv and 0.06 ppbv when plants were 7 weeks of age. These concentrations increased to levels between 35 pptv and 0.14 ppbv when plants were at the 12th week of age.

Stems of the plants were stroked with the intention to damage the trichomes located on the stem. This intention was achieved because visual assessment confirmed that most trichomes located on the stems were broken as a result of the strokes.

An increase in concentration of all mono- and most sesquiterpenes was observed after stroking of the plants. The relative increase (concentration after stroking / concentration before stroking) was up to 60-fold for the monoterpene β-phellandrene. A typical increase of three monoterpenes and the sesquiterpene β-caryophyllene is provided in Table 4.2.

Table 4.2 Relative increase (concentration after stroking / concentration before stroking) of three monoterpenes (β-phellandrene, 2-carene, α-pinene) and the sesquiterpene β-caryophyllene in greenhouse atmosphere after stroking the stems of 60 tomato plants.*

	Relative increase (Concentration after / Concentration before stroking)					
Compounds	7 Weeks	8 Weeks	9 Weeks	10 Weeks	11 Weeks	12 Weeks
β-phellandrene	57.0 (18.1)	59.8 (50.8)	36.9 (9.5)	48.0 (24.8)	35.3 (18.6)	32.8 (13.6)
2-carene	54.5 (20.9)	64.4 (51.3)	39.7 (13.7)	52.4 (13.7)	38.3 (18.0)	40.4 (22.6)
α-pinene	26.9 (14.8)	21.8 (7.12)	10.9 (4.7)	26.2 (3.8)	15.8 (1.1)	19.3 (7.5)
β-caryophyllene	4.7 (2.3)	7.1 (3.2)	5.9 (0.8)	7.7 (2.2)	7.6 (2.8)	5.5 (3.3)

* Results are shown for plants between 7 and 12 weeks old. Means and standard deviations are presented for three independent replicates.

Besides the relative increase, also the absolute increase in concentration of plant-emitted VOCs was studied. The effect of stroking on the absolute concentration of the monoterpene β-phellandrene, and the sesquiterpene β-caryophyllene is shown in Fig. 4.2.

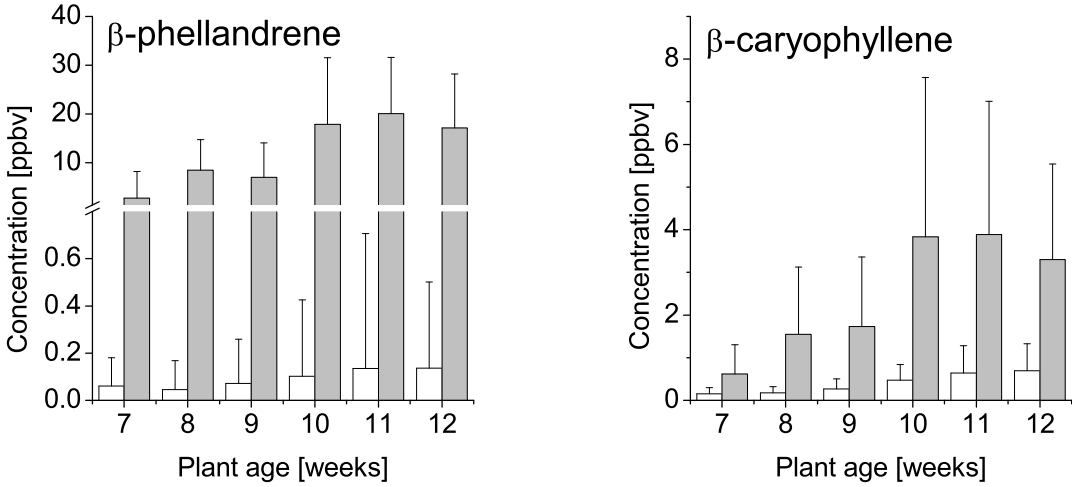

Fig. 4.2 Concentrations of the monoterpene *β*-phellandrene, and the sesquiterpene *β*-caryophyllene in greenhouse atmosphere before (white bars) and after stroking (grey bars) of the plants. Means and standard deviations are presented for 3 independent replicates. Note the different y-axes.

To determine whether the increase in concentration directly after stroking persisted, air samples were collected at 12 h after stroking. Results showed that the concentration decreased to values near to the concentration before stroking (data not shown).

In contrast to the large number of detected mono- and sesquiterpenes, LOX products were undetected in the greenhouse atmosphere before stroking of the plants. The LOX products were also undetected in any of the replicates after stroking of the plants. In addition to the numerous mono- and sesquiterpenes, the phenolic compound methyl salicylate, and trace level amounts of the homoterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were detected in greenhouse atmosphere before stroking the plants. Stroking the plants did not result in substantial increased concentrations of methyl salicylate (Fig. 4.3). Also the concentration of TMTT did not increase after stroking the plants. However, TMTT concentration could not always be quantified since the corresponding signal-to-noise ratio (s/n) was often too low (s/n < 10). In contrast to all other sesquiterpenes detected in greenhouse atmosphere, stroking the plants did not result in substantial increased concentrations of the sesquiterpene *α*-copaene (Fig. 4.3).

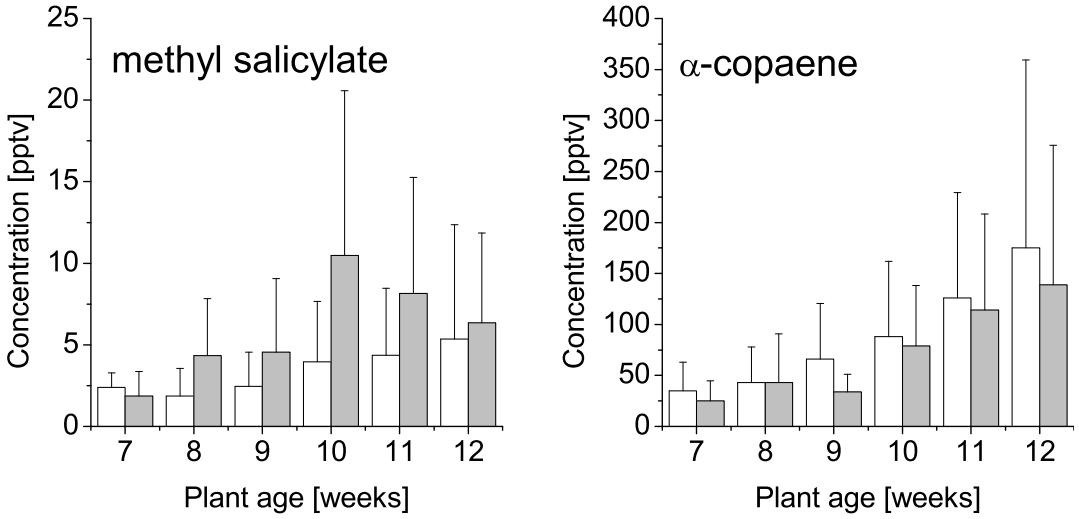

Fig. 4.3 Concentrations of methyl salicylate and α-copaene in the greenhouse atmosphere before (white bars) and after stroking (grey bars) of the plants. Means and standard deviations are presented for three independent replicates. Note the different y-axes.

In addition to the concentration of individual VOCs, also the fractional composition of the VOC mixture was studied. The analysis of air samples collected before damage of the plants revealed that this composition remained nearly constant throughout the growing period. This was indicated by the almost perfect linear relationship between the concentrations of most individual VOCs before damage (Fig. 4.4A and Fig. 4.4C). After stroking, the fractional composition of the plant VOC mixture slightly changed. For some compounds the relation was not affected by the damage (compare Fig. 4.4A with Fig. 4.4B) while for β-phellandrene and β-caryophyllene the ratio after trichome damage increased more then six-fold (compare slopes given in Fig. 4.4C and Fig. 4.4D).

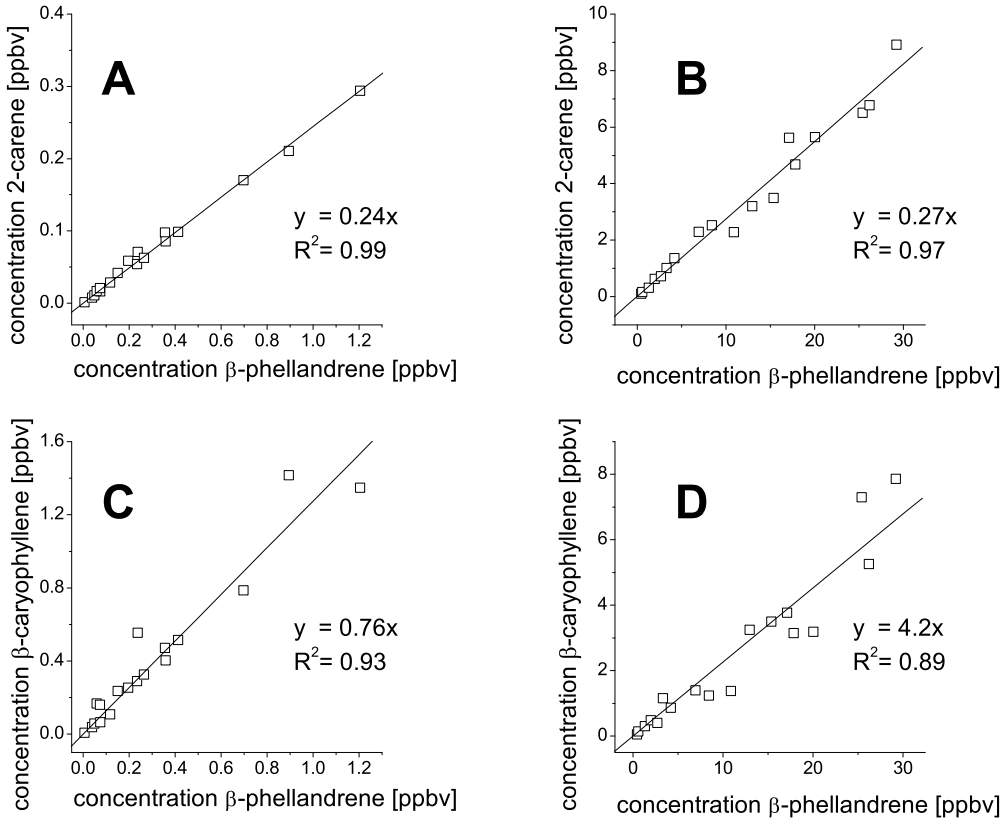

Fig. 4.4 Examples of the linear relation between the concentrations of individual compounds in the mixture of VOCs in greenhouse atmosphere before and after stroking the plants. The data shown represents the relationship for A) *β*-phellandrene and 2-carene before stroking; B) *β*-phellandrene and 2-carene after stroking; C) *β*-phellandrene and *β*-caryophyllene before stroking and D) *β*-phellandrene and *β*-caryophyllene after stroking. Data are derived from three independent replicate studies.

The effect of removing side shoots on VOCs in the greenhouse atmosphere

The quantity and concentration of plant-emitted VOCs detected before removing side shoots resembled that of plant VOCs in the greenhouse atmosphere before stroking the plants. After removal of side shoots, an increase in the concentration of all mono- and most sesquiterpenes was observed. The increases of a monoterpene (*β*-phellandrene) and a sesquiterpene (*β*-caryophyllene) are provided in Table 4.3. Besides the increase in concentration of all mono- and most sesquiterpenes, the compound (*Z*)-3-hexenol appeared in the greenhouse atmosphere after shoot removal at a concentration of 8 to 20 pptv (Table 4.3). In contrast to all other sesquiterpenes, no substantial increase in concentration was observed for the

sesquiterpene α-copaene (Table 4.3). In addition, no substantial increase was observed for the stress-related compounds methyl salicylate, and TMTT (Table 4.3).

Table 4.3 The effect of shoot removal on the concentrations of β-phellandrene, β-caryophyllene, (*Z*)-3-hexenol, α-copaene, methyl salicylate, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) in greenhouse atmosphere.*

	Concentrations: **Before shoot removal [pptv] → After shoot removal [pptv]**		
Compounds	Replicate No. 1	Replicate No. 2	Replicate No. 3
β-phellandrene	46 → 1212	198 → 2883	552 → 8445
β-caryophyllene	76 → 2640	174 → 2534	602 → 9629
(*Z*)-3-hexenol	0 → 10	0 → 8	0 → 20
α-copaene	4 → 10	13→ 9	28 → 32
methyl salicylate	2 → 6	6 → 5	15 → 11
TMTT	23 → 50	179→ 149	63 → 107

* Data are derived from three independent replicate studies.

The effect of fruit picking on VOCs in the greenhouse atmosphere

The quantity and concentration of plant VOCs detected before fruit picking resembled those of plant VOCs in the greenhouse atmosphere before stroking the plants. Directly after fruit picking, all mono- and most sesquiterpenes showed a relative increase in concentration up to 25-fold. The increases of a monoterpene (β-phellandrene) and a sesquiterpene (β-caryophyllene) are provided in Table 4.4. The LOX product (*Z*)-3-hexenol was not detected after fruit picking and no substantial increase in concentration was observed for the stress-related compounds α-copaene, methyl salicylate, and TMTT (Table 4.4).

Table 4.4 The effect of fruit picking on the concentrations of *β*-phellandrene, *β*-caryophyllene, (*Z*)-3-hexenol, *α*-copaene, methyl salicylate, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) in greenhouse atmosphere.*

	Concentrations: **Before fruit picking [pptv] → After fruit picking [pptv]**		
Compounds	Replicate No. 1	Replicate No. 2	Replicate No. 3
β-phellandrene	267 → 4221	627 → 16474	668 → 11583
β-caryophyllene	256 → 1022	349 → 3371	559 → 1668
(*Z*)-3-hexenol	0 → 0	0 → 0	0 → 0
α-copaene	84 → 110	98→ 133	182 → 310
methyl salicylate	2 → 6	6 → 5	15 → 11
TMTT	22 → 10	5→ 2	12 → 7

* Data are derived from three independent replicate studies.

DISCUSSION

Pathogen attack and herbivore infestation have a major impact on plant health. In a model study, these two plant health issues were simulated to study whether plant health can be monitored at greenhouse scale through the analysis of VOCs in greenhouse atmosphere. To simulate pathogen attack and herbivore infestation, we repeatedly stroked the plants and removed the side shoots.

As a result of stroking, the trichomes located on the stems were damaged. These trichomes store, in their interior, considerable amounts of mono- and sesquiterpenes (Buttery *et al.*, 1987). It is therefore likely that damage to these trichomes is responsible for the increase in concentration of most terpenes in the greenhouse atmosphere after stroking (Table 4.2). With each consecutive week, the absolute increase in concentration of all mono- and most sesquiterpenes became larger after stroking of the crop as shown by the example in Fig. 4.2. This result might best be explained by the growth of the plants because plant-growth results in an increase in the total number of trichomes located per stem and thus the amount of stored compounds that readily volatilise after damaging these pools. However, also the history of exposure to the repeated damage might have affected the subsequent plants' response (Bruce *et al.*, 2007).

Detection of a sudden increase in terpene concentration as a result of damage of trichomes provides an interesting approach to monitor plant health because several laboratory studies demonstrated increased emissions as a consequence of trichomes damage because of pathogen infection and herbivore infestation (Jansen *et al.*, 2009c, Loughrin *et al.*, 1994, Maes and Debergh, 2003).

Also, the change in the ratio between the concentrations of individual compounds after stroking could provide an approach to detect stress-induced trichome damage in greenhouses. This second approach is probably more straightforward since relative measurements are, in general, easier to perform. But, also temperature has a significant effect on the emission of compounds from storage pools such as trichomes (Noe *et al.*, 2006). As greenhouse temperatures will fluctuate in horticultural practice, this will have to be taken into account when correlating increased terpene concentrations in greenhouse atmosphere to trichome damage.

The increase in concentration of all mono- and most sesquiterpenes after removing the side shoots can likely be attributed to the damage of trichomes as a result of this treatment. In contrast to the large number of detected mono- and sesquiterpenes, LOX products were undetected in the greenhouse atmosphere before stroking of the plants. This implies that undamaged tomato plants do not emit substantial amounts of LOX products which coincide with the findings and results in Deng *et al*. (2005), Vercammen *et al*. (2001), and Wei *et al*. (2007). After stroking of the plants, LOX products were also undetected, suggesting that stroking does not result in significant breakdown of cell membrane lipids.

The lack of increase in concentrations of methyl salicylate and TMTT after stroking the plants strongly suggests that these two compounds are not stored in high amounts in trichomes of tomato. For methyl salicylate, this was already shown by the work of van Schie *et al*. (2007). In contrast to our findings, other researchers observed increased emissions of methyl salicylate and TMTT as a result of damage to tomato plants. For example, damage of tomato plants as a result of the tobacco mosaic virus resulted in an increase in the emission of methyl salicylate (Deng *et al.*, 2004b). An increase in the emission of methyl salicylate was also observed after caterpillar (*Spodoptera littoralis*) feeding on tomato (Vercammen *et al.*, 2001).

Furthermore, an increase in concentration of both methyl salicylate and TMTT, was reported for spider mite (*T. urticae*)-damaged tomato plants (Dicke *et al.*, 1998, Kant *et al.*, 2004). In the literature, increased methyl salicylate and/or TMTT emissions were also reported upon damage of several other plant species. For instance, Kunert *et al*. (2002) reported a significant increase in the emission of TMTT after the onset of aphid-damage of paprika plants (*Capsicum annuum*). Also Herde *et al*. (2008) reported an increase of TMTT and also methyl salicylate, after herbivore damage of *Arabidopsis*, whereas Röse *et al*. (1996) reported a systemically increased emission of TMTT and methyl salicylate after damage of cotton (*Gossypium hirsutum* L.), in which case the increase was not only observed at the site of damage but also at undamaged leaves.

Why did the concentration of methyl salicylate and TMTT not increase after stroking the tomato plants? We provide three possible directions that explain, at least partially, the absence of methyl salicylate and TMTT after plant damage as a result of stroking the plants. A first explanation for the absence in increase of methyl salicylate and TMTT emission after this type of plant damage is related to time scale. Several studies reported a time period in the order of some hours up to several days between the onset of plant damage and the increased emission of certain VOCs. For example, Vercammen *et al*. (2001) reported a time period of 12 h between the onset of caterpillar damage of tomato plants and the increased emission of methyl salicylate. Such delay might also occur after damage of tomato plants as a result of stroking. Nevertheless, we could not determine if such time delay exists because we only collected air samples directly after the damage took place. The second explanation is also related to time scale. Whereas stroking is an instantaneous action, pathogen infection and herbivore infestation are sustained for several hours or days. This effect was studied by Mithöfer *et al*. (2005) who demonstrated that lima bean plants (*Phaseolus lunatus*) emitted different number and amounts of VOCs in case of continuously sustained damage compared to wounding that was set only once. Continuous sustained damage is not limited to biotic stresses such as pathogen infection and herbivore infestation. Continuous enclosure of plants (a prerequisite for analysing plant emitted VOCs in laboratory), is probably also stressful for plants since emissions of methyl salicylate from untreated tomato plants also increased (Kant *et al.*, 2004). A third explanation for the absence in increase of methyl salicylate and TMTT emission after stroking is related to chemical signalling. It is believed that herbivore-specific signals such as secretions play an important role in activating the systemic release of plant

VOCs (Arimura *et al.*, 2005, Paré and Tumlinson, 1997). Also pathogen-induced signals might play a role in activating the systemic emission of plant VOCs. Neither herbivore- nor pathogen-induced signals are involved in plant damage due to stroking only. The lack of such signals may thus provide clarification for the absence in increase of methyl salicylate and TMTT. In summary, reported conditions under which methyl salicylate and TMTT emissions increased from plants were: (a) an increase in the time scale of measurements, (b) the application of a continuous type of stress (*e.g.* herbivore- or pathogen- induced stress), and (c) the involvement of herbivore- or pathogen-derived signals. However, we cannot exclude the possibility that these conditions have to act simultaneously in order to induce increased methyl salicylate and TMTT emissions from plants.

With regard to the absence of increase in the concentration of *α*-copaene after stroking (Fig. 4.3), we believe that this compound is also not stored in high amounts in trichomes of tomato. It is important to know whether an increase in *α*-copaene emission depend on time scale, and/or requires herbivore- or pathogen-derived signals because this compound was emitted by detached tomato leaves upon *B. cinerea* infection (Thelen *et al.*, 2006) and may thus provide a cue to detect this harmful pathogen in tomato producing greenhouses.

Directly after shoot removal, the compound (*Z*)-3-hexenol appeared in the greenhouse atmosphere (Table 4.3). This compound is one of the LOX products formed after disruption of plant tissue (Matsui, 2006). LOX products mainly consist of a group of C_6-alcohols, aldehydes and acetates also observed after mechanical damage of numerous plant species (Fall *et al.*, 1999, Loreto *et al.*, 2006, Schütz *et al.*, 1997). In addition to mechanical damage, also pathogen inoculation and herbivore infestation result often in the release of LOX products from many plants species including tobacco (*Nicotiana tabacum*), peanut (*Arachis hypogaea*), and lima bean (*P. lunatus*) (Cardoza *et al.*, 2002, De Moraes *et al.*, 2001, Mithöfer *et al.*, 2005). Emission of the LOX product (*Z*)-3-hexenol also occurred after herbivore damage of tomato plants (Deng *et al.*, 2005, Wei *et al.*, 2007) and inoculation of tomato plants with the fungal pathogen *B. cinerea* (Jansen *et al.*, 2009c). A sudden increase in the concentration of (*Z*)-3-hexenol would thus provide a cue for the increased risk for outbreak of these types of plant stress in tomato-producing greenhouses.

In contrast to most terpenes, shoot removal did not result in an increase in concentration of the stress-related compounds methyl salicylate and TMTT (Table 4.3). This contradicts with other studies that established a direct relation between damage of tomato plants as a result of biotic stresses and the increased emissions of methyl salicylate and/or TMTT (Deng *et al.*, 2004b, Dicke *et al.*, 1998, Kant *et al.*, 2004, Vercammen *et al.*, 2001, Wei *et al.*, 2007).

Why did the concentration of methyl salicylate and TMTT not increase after shoot removal? Earlier in this paper, we discussed three possible directions that explain, at least partially, the absence of methyl salicylate and TMTT after plant damage as a result of stroking the plants. These explanations are also applicable for plant damage as a result of shoot removal.

The large increase in concentration of terpenes after fruit picking was attributed to the damage of trichomes during harvest. It is beyond the scope of this study, but such large bursts of emissions after harvest are likely to be common in agricultural practice and might therefore contribute to a higher VOC load of the troposphere (Davison *et al.*, 2008). The absence of LOX products in this case implies that fruit picking does not result in significant breakdown of cell membrane lipids. The lack of increase in the concentration of the stress-related VOCs α-copaene, methyl salicylate and TMTT supports that rejection of ripe fruits is a standard feature of tomato plants and consequently does not results in excessive plant stress.

Based on this study, we cannot ascertain whether a continuously sustained type of damage such as pathogen infection or herbivore infestation will result in an increase in concentration of methyl salicylate, TMTT and/or α-copaene in our experimental greenhouse. The most practical way to address this is the analysis of plant-emitted VOCs after the introduction of a pathogen or herbivore infestation in this greenhouse, which is our next step.

Detecting methyl salicylate, TMTT and/or α-copaene emissions might offer a novel approach to reveal plant stress in a greenhouse if these VOCs are indeed closely linked to herbivore and/or pathogen infestation. However, it should be noted that α-copaene, methyl salicylate, and TMTT emission from tomato are also light dependent (Farag and Paré, 2002, Maes and Debergh, 2003). As light will fluctuate in a greenhouse, this will have to be taken into account when correlating increased α-copaene, methyl salicylate or TMTT concentrations in the greenhouse atmosphere to any type of plant stress.

Our strategy was to simulate pathogen attack and herbivore infestation because these types of stressors are difficult to control and might fully destroy the crop within a short period that does not allow any replicate measurements. We addressed that by repeatedly stroking of the plants and removing the side shoots. Plants were stroked to simulate trichome damage and side shoots were removed to simulate cell membrane damage. It is uncertain whether these two treatments accurately reflect pathogen attack and herbivore infestation since the time scales and also pathogen- and herbivore-specific signals might play an important role in the induction of plant VOCs. An alternative approach would have been to spray the plants with wound signal chemicals such as jasmonic acid, or salicylic acid. These chemicals are known to simulate herbivore- and pathogen-induced emissions of plants (e.g. van Kleunen *et al.*, 2004). But, also these chemicals can only partly simulate the effect of herbivores and/or pathogens.

An important question related to monitoring plant health through analysis of VOCs is the specificity of the signals. A specific signal would allow a particular treatment such as the use of appropriate amounts of insecticides in case of herbivore specific signals. Furthermore, a specific signal would allow us to discriminate between crop damage because of regular crop activities such as harvest and crop damage because of harmful plant health issues. Methyl salicylate and TMTT emissions might offer some specificity because concentrations of these two VOCs did not increase after instantaneous damage of trichomes and cell membranes because of stroking, removing side shoots, and fruit picking, while increased concentrations of these VOCs have been reported frequently after pathogen- and herbivore-induced stresses. Also trichome damage- and cell membrane damage-related VOCs might be used as cues, but only during time periods without greenhouse activities that cause trichome and/or cell membrane damage as well (*e.g.* harvest and shoot removal). An appropriate period, without any greenhouse activities seems more suitable in that respect, for example during night-time. A sudden increase in concentrations of trichome damage- and/or cell membrane damage-related VOCs can then only give a hint because neither trichome nor cell membrane damage is specific to a particular herbivore infestation or pathogen infection. However, most herbivore and pathogens inflict quite different types of plant damage. For example, infection of tomato plants with the fungus *B. cinerea* is usually restricted to the stem where it can cause stem rot (Shtienberg *et al.*, 1998). In contrast, spider mites (*T. urticae*) feed on cell content of

mesophyll cells, hereby causing wound sites spread out over the whole leaf area of tomato (Kant *et al.*, 2004). It can be expected that the type of damage inflicted by the type of stress has a large effect on the extent of trichome and cell membrane damage. Then, trichome damage- and cell membrane damage-related VOCs have the potential to provide information about the type of damage but perhaps not on the particular causal agent of the plant health issue.

Results employed in this study were based on offline analyses using GC-MS. This delicate instrument is often restricted to laboratory use. For application in greenhouses, on-site analyses are probably required. More robust GC-MS systems might then be considered because such a system proved to be capable of detecting plant emitted VOCs at the low pptv level (Karl *et al.*, 2008). Recent advances in biosensor technology might provide an alternative approach. For example, insect antennae are highly sensitive to certain compounds, such as plant volatiles (Weißbecker *et al.*, 2004). Another trend in plant volatile analysis is the development of fast and sensitive systems based on electronic noses (Laothawornkitkul *et al.*, 2008). The appropriate system for application in a large-scale greenhouse is under debate, and selection depends on costs and benefits.

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CHAPTER 5

The effect of *Botrytis cinerea* on the concentration of volatiles in a small-scale greenhouse

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INTRODUCTION

Despite all efforts to keep plants healthy, phytopathogens still cause serious economic damage in greenhouse cultivation (Elad, 1999). Early detection of pathogen infections would enable better management and control. Crops are therefore subjected to regular inspections to look for symptoms to avoid too much damage. However, symptoms are then often overlooked since many pathogenic problems emerge at the abaxial side of leaves or on stem parts that are hidden by the foliage. For example, infections by some plant pathogens result in visual symptoms which are restricted to the stem. When these symptoms remain unnoticed, the infection may lead to stem-rot which results in low yields and even plant death (Shtienberg *et al.*, 1998). Researchers have therefore sought new ways to detect such hidden symptoms of pathogen infections. This could enable a grower to take early action, preventing pathogen spread, and further damage by controlling the problem right at the source. A proposed concept to direct a grower to the presence of a pathogen infection is based on the detection of pathogen-induced emissions of volatile organic compounds (VOCs) from plants (Schütz, 2001).

This concept was evaluated in a collaboration project between Wageningen University, Plant Research International, and Research Centre Jülich. The aim of that project was to assess whether plant-emitted VOCs can be used to direct a grower to the presence of a pathogen infection at greenhouse scale. In this project, we used tomato (*Lycopersicon esculentum* Mill) and the grey mould pathogen *Botrytis cinerea* as model organisms to investigate this concept.

Based on the results of a laboratory-scale study, Jansen *et al*. (2009c) reported the detection of plant-emitted C_6-compounds after the inoculation of tomato plants with *B. cinerea* spores. The detection of these C_6-compounds was attributed to the damage of cell membranes. These cell membranes contain C_{18}-fatty acids which are converted enzymatically into volatile C_6-compounds upon damage of cell membranes. Pathogens have the ability to damage cell membranes (Levin, 1976) and the detection of C_6-compounds could thus be explained. Similar to the detection of C_6-compounds at laboratory scale, C_6-compounds were also detected at greenhouse scale (Jansen *et al.*, 2009b). In the latter study, these type of comounds were detected after removing the side shoots from tomato plants. The first objective of the present study was to determine whether these C_6-compounds are also detectable at greenhouse

scale after inoculation of tomato plants, and if so, to determine the time course of the concentrations of these compounds.

The laboratory scale study described in Jansen *et al*. (2009c) also reported an increase in the concentration of several mono- and a few sesquiterpenes after the inoculation of tomato plants with *B. cinerea* spores. Such an increase in concentration of monoterpenes and sesquiterpenes is most likely the result of damage to glandular trichomes. These glandular trichomes contain monoterpenes and sesquiterpenes in their interior which readily volatilize when the trichome is damaged. Pathogens have the ability to damage trichomes (Gibson, 1971) and the increase in concentration of these terpenes could thus be explained. Similar to the increased concentration of terpenes at laboratory scale, terpene concentrations also increased at greenhouse scale (Jansen *et al.*, 2009b) after picking fruits and removing side shoots from, and after stroking the stems of tomato plants. The second objective of the present study was to determine whether the concentration of these terpenes also increases after inoculation of tomato plants at greenhouse scale.

Finally, Jansen *et al*. (2009c) reported a gradual increase in concentration of methyl salicylate and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) after the inoculation of tomato plants with *B. cinerea* spores. These two substances are regarded as volatile plant hormones (Arimura *et al.*, 2005). The emission of these two compounds is generally believed to increase several hours, or days, after the onset of various types of biotic and abiotic stress in various plant-species (*e.g.* Kant *et al.*, 2004). In contrast to the increase in concentration of most terpenes, the concentration of methyl salicylate and TMTT did not increase at greenhouse scale, neither after picking fruits and removing the side shoots nor after stroking the stems (Jansen et al. 2009b). The third objective of the present study was to determine whether the concentration of methyl salicylate and/or TMTT increases after inoculation of tomato plants at greenhouse scale.

MATERIALS AND METHODS

Plant material and inoculation

Seeds of tomato plants (*Lycopersicon esculentum* Mill.) of the cultivar Moneymaker, were germinated in a standard greenhouse at 20°C and 50% relative humidity (RH). When plants were about seven weeks old, 60 plants were transferred to a small experimental greenhouse. Plants were 14 weeks old and 2 m in height, when they were spray-inoculated with a spore suspension on the adaxial leaf surfaces. The suspension consisted of 1 L filter sterilized water supplemented with 6 g potato dextrose medium and 5.4×10^8 *Botrytis cinerea* spores. Each plant was inoculated with 15 mL of this aqueous suspension on the 23th of June, 2008 at 19:00 h.

Monitoring visual symptoms of *Botrytis cinerea* infection

Ten plants were randomly selected and one leaf per selected plant, randomly located at mid-canopy height, was labelled before inoculation of the plants. Pictures of the adaxial side of these ten leaves were taken at 0, 24, 48, and 72 h after inoculation (HAI). The individual leaves were classified based on the visual symptoms depicted on these pictures. The leaf was classified as “no symptoms” in case no effect of the inoculation was visible. The leaf was classified as “mild symptoms” in case small and restricted necrotic spots were visible and the leaf was classified as “severe symptoms” in case large and non-restricted necrotic regions occurred.

Greenhouse equipment and climate control

The floor area of the greenhouse was 44 m^2 and the total volume including the basement underneath was 270 m^3. A fan located in the basement was used to maintain a constant internal air circulation of 20×10^3 m^3 h^{-1}. Electrical heating and direct mechanical cooling situated in the basement controlled temperature and humidity. The temperature was set at 22°C/16°C ±1.0°C day/night and no supplementary light was used. The RH inside the greenhouse was set at 70%/90% ±5% day/night. The temperature, RH and light intensity inside of the greenhouse were recorded with a time interval of 5 min.

Air sampling in the greenhouse

A sequential sampler was used to purge air from the greenhouse through stainless steel cartridges packed with 200 mg of Tenax-TA 20/35. Air was sucked through these cartridges at 300 mL min^{-1} for 60 min (total volume of 18 L for each cartridge). The greenhouse air was sampled with a 1 h time interval until 72 HAI. After sampling, the cartridges were capped and transferred to the laboratory for analysis.

Identification and quantification of the plant-emitted VOCs

The identification and quantification of plant-emitted VOCs in the sample was performed using gas chromatography and mass spectrometry. A detailed description of this instrument, the measurement method, and the data analysis has been described elsewhere (Jansen *et al.*, 2009b).

RESULTS

Climate control

The time courses of the RH and light intensity inside the greenhouse were similar in- between the three days following inoculation (Fig. 5.1A and Fig. 5.1B). The time course of the temperature inside of the greenhouse was similar in-between those three days (Fig. 5.1C).

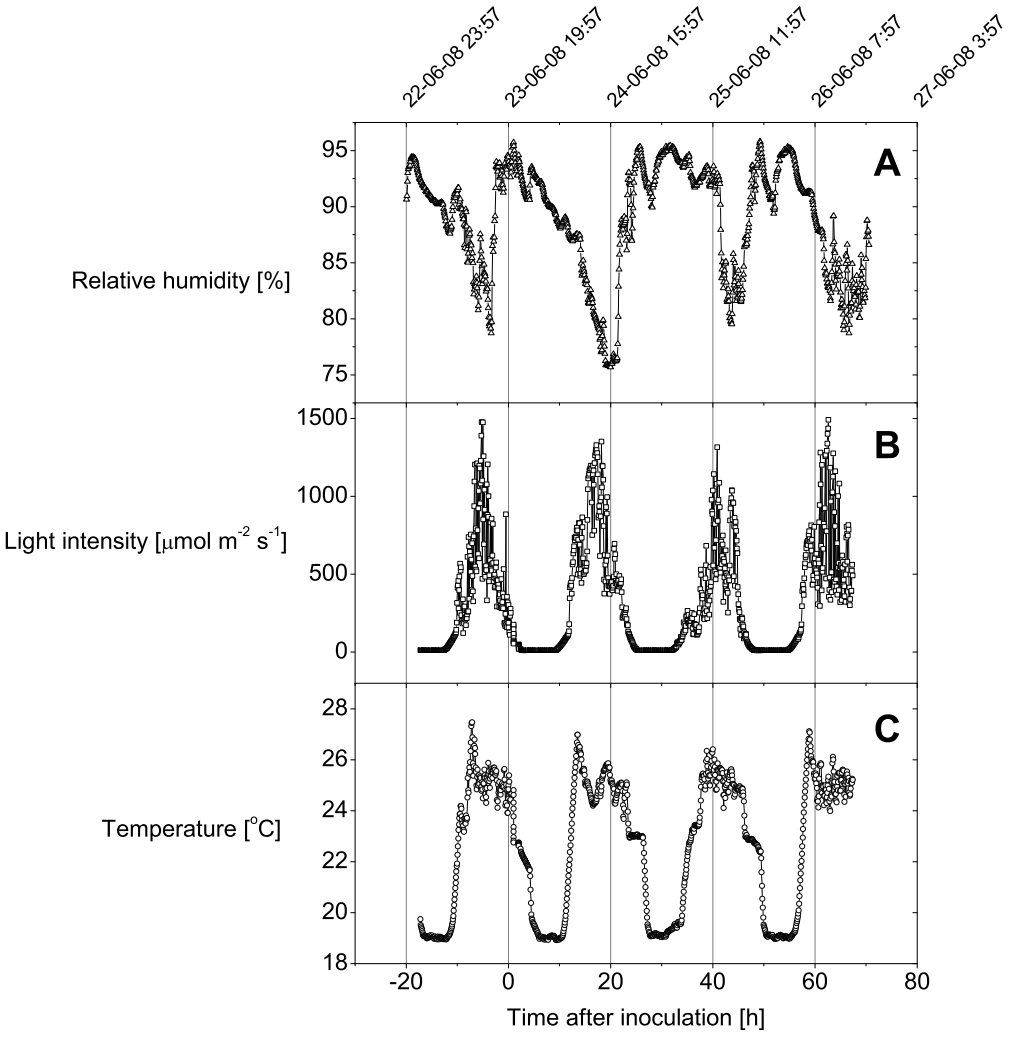

Fig. 5.1 The time course of the (A) relative humidity (-Δ-), (B) light intensity (-□-), and (C) temperature (-○-) inside the greenhouse.

Monitoring of visual symptoms

Two pictures, both taken at 72 HAI are provided in Fig. 5.2 to demonstrate the differences in visual symptoms at a certain time point. These pictures show a leaf which was classified as "mild symptoms" and a leaf which was classified as "severe symptoms". Based on the pictures taken at 0 HAI, all ten leaves were classified as "no symptoms". The pictures taken at 24 HAI, showed one leaf with some small necrotic spots. At 48 HAI, the size of these spots increased and this leaf was then classified as "severe symptoms". At 48 HAI, two additional leaves showed small necrotic spots, then classified as "mild symptoms". Based on the pictures taken at 72 HAI, three leaves were classified as "mild symptoms" and two leaves were classified as "severe symptoms". Fig. 5.3 summarizes these classification results.

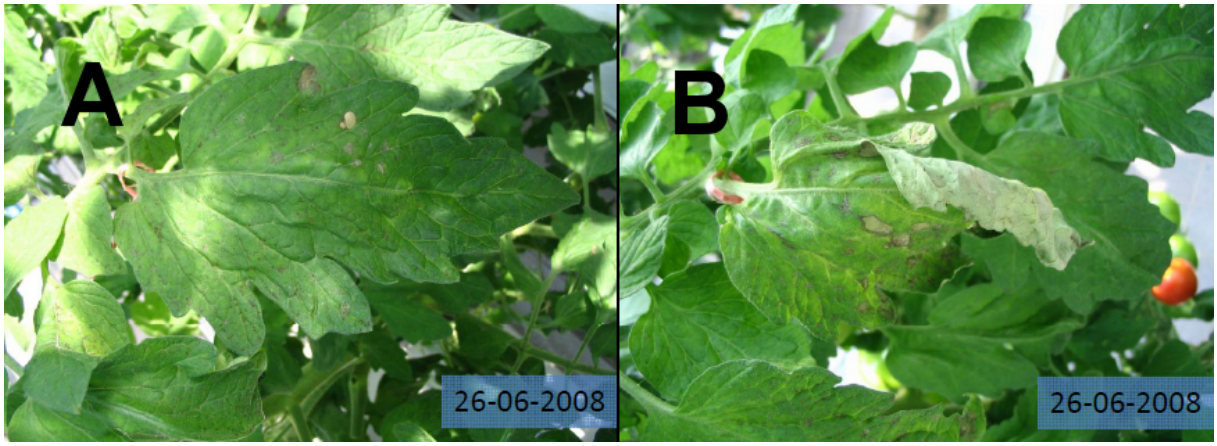

Fig. 5.2 Pictures of leaves classified as (A) "mild symptoms", and (B) "severe symptoms". Both pictures were recorded at 72 h after inoculation.

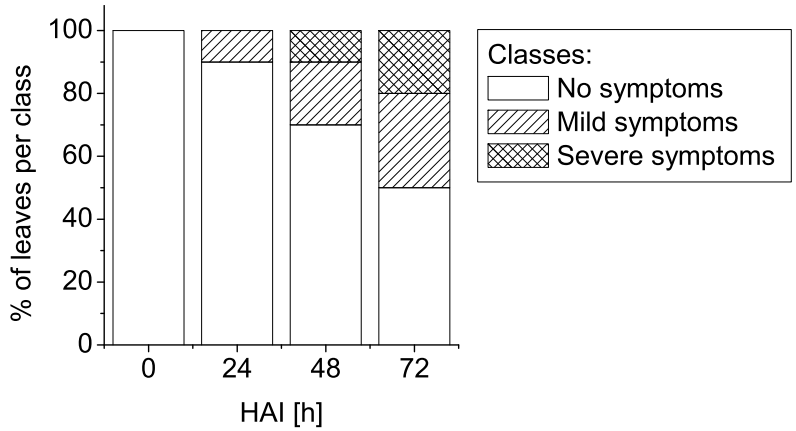

Fig. 5.3 Classification of ten randomly selected leaves at 0, 24, 48, and 72 h after the inoculation (HAI) of tomato plants with *Botrytis cinerea* spores.

Concentration of plant-emitted VOCs

In the greenhouse air samples, C_6-compounds were undetected while at least twelve monoterpenes could be detected. The concentrations of all monoterpenes were relatively high at two HAI and then decreased to a nearly constant level. As a representative of the monoterpenes, Fig. 5.4A shows the time course of β-phellandrene. At least four sesquiterpenes were detected per sample. The concentrations of most sesquiterpenes were relatively high at two HAI and then decreased to a nearly constant level. As a representative of the sesquiterpenes, Fig. 5.4B shows the time course of β-caryophyllene. In contrast to the almost constant concentration of most sesquiterpenes, the concentration of the sesquiterpene α-copaene fluctuated with the day/night rhythmicity (Fig. 5.4B). In addition to the large number of terpenes, the ester-substituted phenol, methyl salicylate, was detected in all samples. At 0 HAI, the concentration of methyl salicylate was ~10 pptV. A 10-times and 3-times increase in the concentration of methyl salicylate at respectively 32 and 34 HAI was observed (Fig. 5.4C). The homoterpene TMTT was also detected in all samples. The concentration of this compound remained almost constant at the low pptV level (data not shown).

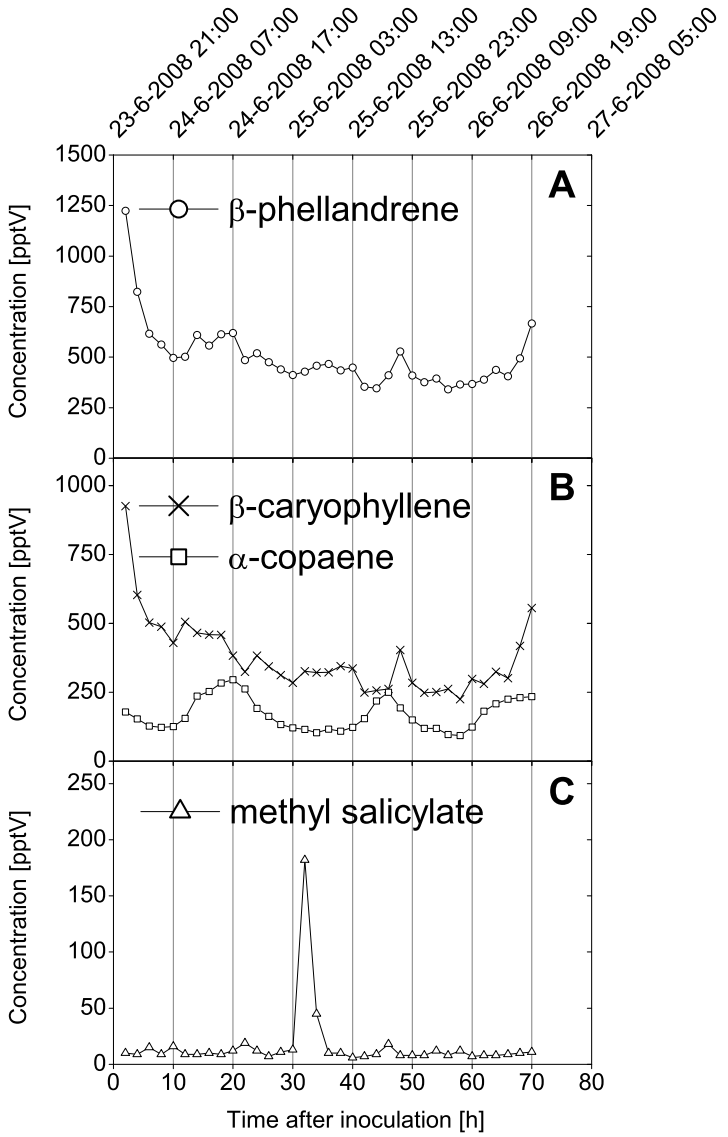

Fig. 5.4 The time course of the concentration of (A) the monoterpene *β*-phellandrene (-○-), (B) the sesquiterpenes *β*-caryophyllene (-×-) and *α*-copaene (-□-), and (C) methyl salicylate (-Δ-) after the inoculation of tomato plants with *Botrytis cinerea* spores.

DISCUSSION

In this section, we discuss the effect of *B. cinerea* infection on the measured concentrations of plant-emitted volatiles in the greenhouse. Besides a pathogen infection, temperature and light have an effect on the emissions of VOCs from tomato (*e.g.* Farag and Paré, 2002). Therefore, an additional effect of temperature and light was expected on the concentrations of plant-emitted VOCs in the greenhouse. However, since the time courses of temperature and light

were similar in-between the three days (Fig. 5.1B and Fig. 5.1C), it is reasonable to ascribe unexpected fluctuations in concentrations to other factors.

The first objective of this study was to determine whether C_6-compounds are detectable after inoculation of tomato plants at greenhouse scale, and if so, to determine the time course of the concentrations of these compounds. In contrast to the large amount of C_6-compounds detected in samples obtained after inoculation of tomato plants at laboratory scale (Jansen *et al.*, 2009c), C_6-compounds were undetected in the samples obtained at greenhouse scale. A first explanation, maybe the low extent of damage to cell membranes which was probably not sufficient to induce detectable concentrations of C_6-compounds in greenhouse air. This is in agreement with the low percentage of leaves classified as "severe symptoms" and "mild symptoms" throughout the experimental period (Fig. 5.3). The relatively mild symptoms may be due to the fact that the tomato plants used in the present study were 14 weeks old. Tomato plants of this age are in general quite resistant and less viable to infection compared to the young plants used in laboratory scale studies. Second, the RH inside the greenhouse was often below 90% within the first 24 HAI (Fig. 1A). This might have caused the low infection level since the RH should be maintained at high levels (> 95%) during at least 24 h to establish a serious *B. cinerea* infection. Another explanation for the undetected C_6-compounds is not related to the emission of VOCs, but related to possible loss processes for plant-emitted VOCs. A loss process to consider is the solution of these polar compounds into water bodies that occurred on the glass cover and dehumidifier used for air conditioning.

The second objective of this study was to determine whether the concentrations of mono- and/or sesquiterpenes increases after inoculation of tomato plants at greenhouse scale. The relatively high concentrations of all mono- and most sesquiterpenes at 2 HAI (Fig. 5.4) was likely the result of damage to glandular trichomes because of the large amounts of small droplets hitting the plants. After this initial increase had levelled off, no significant increases in the concentrations of any mono- and/or sesquiterpenes were observed. Probably, the extent of pathogen-induced damage to trichomes was insufficient within this period to induce a significant increase in concentration of mono- and/or sesquiterpenes. The extent of trichome damage and the severity of infection are most likely closely related. The lack of increase in concentration of monoterpenes and sesquiterpenes is therefore in agreement with the low percentage of leaves classified as "severe symptoms" and "mild symptoms". Reasons for

these low percentages were discussed before in this paper. A second explanation for the almost constant concentration of all monoterpenes and most sesquiterpenes might be the relatively low air exchange rate compared to the laboratory set-up described in Jansen *et al.* (2009c); 0.56 exchanges of the greenhouse volume per hour *versus* 3 exchanges of the chamber volume per hour. As a consequence, fluctuations in the concentrations of mono- and sesquiterpenes are levelled out. The absence of fluctuations is unexpected since the 7°C increase in temperature during the day must have increased the emission of mono- and sesquiterpenes from the tomato plants. The only compound of which the concentration fluctuated according to the day/night rhythm was α-copaene. This compound is not stored in trichomes of tomato and its emission has been suggested to be light-dependent (Maes and Debergh, 2003). Likely, the effect of light on the emission of α-copaene is stronger than the effect of temperature on the emission of the other sesqui- and monoterpenes. The day/night fluctuations in the concentration of α-copaene indicate that plant-emitted volatiles reflect time-dynamic plant responses at greenhouse scale.

The third objective of this study was to determine whether the concentration of methyl salicylate and/or TMTT increases after inoculation of tomato plants at greenhouse scale. The 10-fold and 3-fold increase in concentration of methyl salicylate at respectively 32 HAI and 34 HAI suggest a pulsed emission of this volatile plant hormone at that time period. Interestingly, the increase in concentration co-occurred with the onset of light (compare Fig. 5.1 with Fig. 5.4). Maybe, methyl salicylate had accumulated in the stomatal cavity overnight. Opening of the stomata at the onset of light may have induced an emission burst in methyl salicylate. Replicate studies are required to determine whether methyl salicylate is a reliable indicator of a *B. cinerea* infection at greenhouse scale. In contrast to the increase in concentration of TMTT after inoculation of tomato plants at laboratory scale (Jansen *et al.*, 2009c), the concentration of TMTT remained nearly constant at greenhouse scale. Probably, the extent of infection was not sufficient to induce such an increase.

Besides an infection with *B. cinerea*, tomato plants might be challenged with other biotic and/or abiotic stress factors. This aspect highlights an important issue related to the specificity of methyl salicylate emissions from tomato plants. A system that has the opportunity to not only detect a stress, but also to identify the causal agent would be of great value as it would allow deciding on the proper control measure. What makes methyl salicylate less suitable for

this purpose is that increased emissions of methyl salicylate are induced upon different biotic and abiotic stresses of tomato (*e.g.* Deng *et al.*, 2004b, Dicke *et al.*, 1998). On the other hand, the concentration of methyl salicylate did not increase after picking fruits and removing the side shoots from tomato (Jansen *et al.*, 2009b). Hence, the detection of an increase in methyl salicylate concentration might thus direct towards the presence of a *B. cinerea* infection since the diversity of stress factors that occurs in a greenhouse-grown tomato crop is often limited, primarily due to the monoculture and environmental control. It is still unknown whether the detection of an increase in methyl salicylate is sufficient to direct a grower towards the presence of a *B. cinerea* infection of tomato with high degree of certainty.

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CHAPTER 6

A model to predict the effect of *Botrytis cinerea* on the concentration of volatiles in a large-scale greenhouse

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Nomenclature

SR_h	emission rate of a VOC by healthy plants	[mol s^{-1}]
SR_B	emission rate of a VOC by *Botrytis cinerea* infected plants	[mol s^{-1}]
SR_{air_in}	entrance rate of a VOC by air coming into the greenhouse	[mol s^{-1}]
SR_{mat}	emission rate of a VOC by greenhouse material	[mol s^{-1}]
SR_{air_out}	removal rate of a VOC by air coming out of the greenhouse	[mol s^{-1}]
SN_{mat}	adsorption/absorption rate of a VOC by greenhouse materials	[mol s^{-1}]
SN_{water}	transfer rate of a VOC into water bodies	[mol s^{-1}]
SN_{plant}	adsorption/ absorption rate of a VOC by plants	[mol s^{-1}]
SN_{O3}	gas phase reaction rate of a VOC with O_3	[mol s^{-1}]
V	volume of greenhouse	[m^3]
$[VOC]_{gr}$	molar concentration of a VOC in the greenhouse air	[mol m^{-3}]
$[O_3]$	molar concentration of O_3 in the greenhouse air	[mol m^{-3}]
t	time	[s]
n_h	number of healthy tomato plants	[-]
Φ_h	emission flux density of a VOC by healthy plants	[mol m^{-2} s^{-1}]
n_B	number of *Botrytis cinerea* infected plants	[-]
Φ_B	emission flux density of a VOC by *Botrytis cinerea* infected plants	[mol m^{-2} s^{-1}]
A_{plant}	one-sided leaf area per tomato plant	[m^2]
f_{air_in}	volumetric flow rate of air coming into the greenhouse	[m^3 s^{-1}]
$[VOC]_{air\_in}$	molar concentration of a VOC in the incoming air	[mol m^{-3}]
f_{air_out}	volumetric flow rate of air coming out of the greenhouse	[m^3 s^{-1}]
$[VOC]_{air\_out}$	molar concentration of a VOC in the air coming out of the greenhouse	[mol m^{-3}]
k_{mat_air}	mean emissions rate of a VOC by greenhouse materials	[mol m^{-2} s^{-1}]
k_{air_mat}	mean adsorption/absorption rate of a VOC by greenhouse materials	[mol m^{-2} s^{-1}]
A_{mat}	adsorption/absorption area of the greenhouse materials	[m^2]
A_{water}	transfer area of the water bodies	[m^2]
k_{air_water}	transfer coefficient for uptake of a VOC into water bodies	[m s^{-1}]
k_{plant}	mean adsorption/absorption rate of a VOC by plants	[mol s^{-1} m^{-2}]

k_{O3}	rate constant for the reaction of ozone with a VOC	[m^3 mol^{-1} s^{-1}]
$[CO_2]_{gr}$	molar concentration of CO_2 in the greenhouse	[mol m^{-3}]
$[CO_2]_{amb}$	molar concentration of CO_2 in ambient air outside the greenhouse	[mol m^{-3}]

INTRODUCTION

Industries all over the world face ongoing expansion and intensification to serve the needs of a growing population. Greenhouse industry also moves towards large scale systems while small greenhouses are closed (Breukers *et al.*, 2008). The cultivation of crops in these large scale greenhouses is characterized by the monoculture of high value crops at high plant density throughout the entire year. These conditions increase productions per surface area. However, the year round production of one single, high density crop, also provides excellent circumstances to establish and spread pathogens and pests throughout the greenhouse (Elad, 1999, van Lenteren, 2000).

Nowadays, regular human inspection is common practice to monitor crops for the presence of diseases and pests. These inspections must be accurate in large-scale greenhouses since inaccurate inspections allow local disease or pest problems to disperse rapidly over long distances which in turn result in large economic losses. These accurate, on-site inspections of crops are time consuming and require skilled personnel which in-turn leads to high costs. As a result, greenhouse managers want to automate these on-site inspections to limit the demand for manual labour. Thus, expansion and intensification of the greenhouse industry increase the demand for an automated system to monitor greenhouse crops for the presence of diseases and pests. Such a system would facilitate immediate actions and prevent further spread by controlling the problem right at the source.

Researchers have sought efficient ways to monitor greenhouse crops for the presence of diseases and pests. One option is the analysis of air to identify and/or quantify trace level amounts of volatile organic compounds (VOCs) emitted from plants which suffer from pest or disease problems (Schütz and Weißbecker, 2003).

Infections of crops with the pathogenic fungus *Botrytis cinerea* are among the most common cause of reduced yields in greenhouse horticulture (Elad and Stewart, 2004). During 2005-2008, we carried out a broad range of experiments to test whether VOCs can be used as an indicator of the presence of a *B. cinerea* infection in a large scale tomato production greenhouse. In the first phase, we analysed the air surrounding tomato leaves individually enclosed in 300 mL Petri dishes. Results demonstrated that leaf-emitted VOCs can be used as an indicator of the presence of a *B. cinerea* infection at this ultra-small scale (Thelen *et al.*,

2006). In the second phase, we analysed the air surrounding several tomato plants grown in a 1 m^3 chamber. Results of that follow-up study demonstrated that plant-emitted VOCs can be used as an indicator of the presence of a *B. cinerea* infection also at this intermediate scale (Jansen *et al.*, 2009c). In the third phase, we analysed the air surrounding 60 tomato plants, grown in a small, 270 m^3 greenhouse. Results obtained during that phase indicate that crop-emitted VOCs can be used as an indicator of the presence of a *B. cinerea* infection in a small scale greenhouse (Jansen *et al.*, 2009a). The 60 plants grown in the small scale greenhouse were situated on a 42 m^2 floor area. In commercial large-scale greenhouses, many more plants are grown at far larger floor areas. For example, at present, the majority of such large-scale greenhouses in Western European countries, such as the Netherlands, may contain 2×10^4 to 2×10^5 plants and have floor areas which range between 10^4 and 10^5 m^2 (van Henten, 2006). The objective of this study was to determine whether plant emitted volatiles can be used as an indicator of the presence of a *B. cinerea* infection in such a commercial large-scale greenhouse.

To achieve this objective, experiments can be conducted and in principle, such experimental studies provide the most realistic results. However, experimental studies require expensive, well controlled facilities. Moreover, the results obtained under the tested conditions may not be directly applicable for different conditions. Owing to these limitations, mathematical models are increasingly being used to bridge the gap between experimental studies and real world applications. Such model approach is also useful in translating the results obtained in a small-scale greenhouse into the potential application of crop health monitoring in commercial large-scale greenhouses.

MATERIALS AND METHODS

Plant material

The experiments were performed using tomato plants (*Lycopersicon esculentum* Mill) of the cultivar Moneymaker.

Volatile organic compounds

Tomato plants emit different types and amounts of volatiles during infection by *B. cinerea*. The main effects are the burst of lipoxygenase products and the increase in emissions of

monoterpenes, sesquiterpenes, methyl salicylate and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The burst of lipoxygenase products is probably the result of damage to cell membranes. The increase in emissions of monoterpenes and sesquiterpenes is probably the result of damage to glandular trichomes. The increase in emission of the volatile plant hormones methyl salicylate and TMTT is not directly related to cell membrane or trichome damage but probably the result of a systemic plant response as a result of stress (Jansen *et al.*, 2009c). Five volatile organic compounds (VOCs) were investigated in this study: a lipoxygenase product (the C_6-alcohol (*Z*)-3-hexenol), three trichome damage induced VOCs (the monoterpenes *α*-pinene and *α*-terpinene, and the sesquiterpene *β*-caryophyllene), and a volatile plant hormone (the ester-substituted phenol methyl salicylate).

Experimental greenhouse

A schematic illustration of the small scale greenhouse used in this study is presented in Fig. 6.1. This greenhouse has been described in detail by Körner *et al.* (2007). In short, the floor area of the greenhouse is 44 m^2 and the total volume including the basement underneath is 270 m^3. A fan located in the basement was used to maintain a constant internal air circulation of 20×10^3 m^3 h^{-1}. The temperature in the greenhouse was set at 22/16 ±1.0 °C day/night. The relative humidity inside the greenhouse was set at about 70/90 ±5% day/night. An air conditioner, consisting of a heater, cooler, and dehumidifier situated in the basement maintained these temperatures and humidity. The greenhouse was semi closed; it did not contain any windows that could be opened. Air was supposed to come in through gaps in the basement, and go out through gaps in the glass cover. Pure CO_2 was supplied to maintain a CO_2 concentration of 0.015 mol m^{-3}.

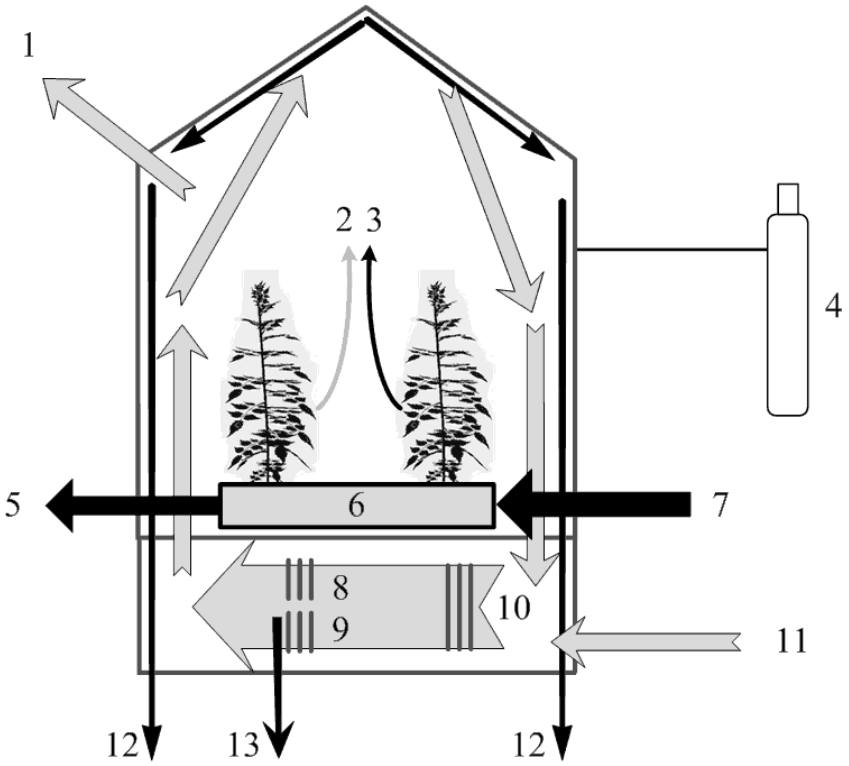

Fig. 6.1 Schematic illustration of the small-scale greenhouse used in this study: (1) air leaving the greenhouse, (2) emission of volatile organic compounds (VOCs) by the plants, (3) transpiration by the plants, (4) CO_2-supply, (5) water leaving the slab, (6) slab, (7) water entering the slab (8) cooler, (9) de-humidifier, (10) heater, (11) air entering the greenhouse, (12) water leaving the gutters, and (13) water leaving the dehumidifier. Grey arrows represent the transport of airborne VOCs, and black arrows represent the transport of liquid water.

Mass transfer model

For this study it was assumed that the air in a greenhouse is perfectly mixed. This means that the concentration of plant emitted VOCs is assumed to be similar at each location within the greenhouse. It was furthermore assumed that the volumetric flow rate of air entering the greenhouse due to forced or natural ventilation was equal to the volumetric flow rate of air leaving the greenhouse. A certain number of healthy plants is assumed emitting VOCs constitutively. Another number of plants is assumed to emit *B. cinerea* induced emissions. The leaf area per plant is assumed to be same.

Four sources and five sinks were considered as most relevant for the mass balance of VOCs in greenhouse air.

The first source was the emission of a VOC by healthy plants. The emission rate of a VOC by healthy plants (SR_h) gives the total amount of the VOC introduced by the plants into the greenhouse air per unit of time. It depends on the number of healthy plants (n_h), on the emission flux density of the VOC by the healthy plants (Φ_h), and on the one-sided leaf area per plant (A_{plant}). This rate is described by Eq. (6.1):

$$SR_h = n_h \cdot \Phi_h \cdot A_{plant} \tag{6.1}$$

The second source was the emission of a VOC by *B. cinerea* infected plants. The emission rate of a VOC by *B. cinerea* infected plants (SR_B) depends on the number of infected plants (n_B), on the emission flux density of the VOC by *B. cinerea* infected plants (Φ_B), and on the one-sided leaf area per plant (A_{plant}). This rate is described by Eq. (6.2):

$$SR_B = n_B \cdot \Phi_B \cdot A_{plant} \tag{6.2}$$

The third source was the introduction of a VOC together with the air entering the greenhouse. For instance, trees nearby a greenhouse may emit some of the VOCs considered in this study, which can then be transferred by wind to the ambient air outside the greenhouse, and can then enter the greenhouse. The introduction rate of a VOC by this process (SR_{air_in}) depends on the volumetric flow rate of this air (f_{air_in}), and the concentration of the VOC in this air ($[VOC]_{air\_in}$). This rate is described by Eq. (6.3):

$$SR_{air\_in} = f_{air\_in} \cdot [VOC]_{air\_in} \tag{6.3}$$

The fourth source was the emission of a VOC by greenhouse materials. For example, glues or epoxies, drying agents in paints, and soft plastics emit significant amounts of VOCs into the surrounding air (*e.g.* Guo, 2002). The emission rate of a VOC by greenhouse materials (SR_{mat}) depends on the emission coefficient of greenhouse materials (k_{mat_air}), and the transfer area of the greenhouse materials (A_{mat}). This rate is given by Eq. (6.4):

$$SR_{mat} = k_{mat\_air} \cdot A_{mat} \tag{6.4}$$

The first sink was the removal of a VOC by air leaving the greenhouse. For instance, open windows at the top of a greenhouse allow air to leave together with the VOCs therein. The removal rate of a VOC by this process (SN_{air_out}) depends on the volumetric flow rate of air leaving the greenhouse (f_{air_out}), and the concentration of the VOC in this air ($[VOC]_{air\_out}$). This rate is described by Eq. (6.5):

$$SN_{air\_out} = f_{air\_out} \cdot [VOC]_{air\_out} \tag{6.5}$$

The second sink was the adsorption/absorption of a VOC by greenhouse materials. This process might be important since many different building materials absorb VOCs (see Yang *et al.*, 2001 and references therein). The adsorption/absorption rate of a VOC by greenhouse materials (SN_{mat}) depends on the adsorption/absorption coefficient of greenhouse materials (k_{mat_air}), and the transfer area of the greenhouse materials (A_{mat}). This rate is described by Eq. (6.6):

$$SN_{mat} = k_{air\_mat} \cdot A_{mat} \tag{6.6}$$

The third sink was the adsorption and/or absorption of a VOC by plants. This sink can be the result of adsorption of VOCs on the plant cuticle as well as absorption through the stomata (Riederer *et al.*, 2002). The adsorption/absorption rate of a VOC by plants (SN_{plant}) depends on the number of healthy plants (n_h), the number of *B. cinerea* infected plants (n_B), the adsorption/absorption coefficient of plants (k_{plant}), and the one-sided leaf area per plant (A_{plant}). This rate is described by Eq. (6.7):

$$SN_{plant} = (n_h + n_B) \cdot k_{plant} \cdot A_{plant} \tag{6.7}$$

The fourth sink was the degradation of VOCs through gas-phase reactions. Such gas-phase reactions between plant-emitted VOCs and hydroxyl radicals (OH), nitrate radicals (NO_3) and ozone (O_3) are common in the lower atmosphere (Atkinson and Arey, 2003, Canosa-Mas *et al.*, 2002). Degradation of VOCs through gas-phase reactions with O_3 were regarded as most important. The removal rate of a VOC by gas-phase reaction with O_3 (SR_{O3}) depends on the

volume of the greenhouse (V), the concentration of the VOC in the greenhouse air ($[VOC]_{gr}$), the concentration of O_3 in the greenhouse air ($[O_3]$) and the rate constant for the reaction of O_3 with the VOC (k_{O3}). This dependence is described by Eq. (6.8).

$$SN_{O3} = V \cdot [VOC]_{gr} \cdot [O_3] \cdot k_{O3} \quad (6.8)$$

The fifth sink was the transfer of a VOCs into water bodies. This may occur if a VOC is solved into liquid water which had been in contact with airborne VOCs such as condensation on the inner side of the greenhouse cover. This sink seems relevant for VOCs which are soluble in water such as methyl salicylate (Henry's law coefficient = 0.33 mol/(m^3 Pa); Karl *et al.*, (2008)) and (*Z*)-3-hexenol (Henry's law coefficient = 0.75 mol/(m^3 Pa); Atlan *et al.* (2006)). The transfer rate of a VOC into water bodies (SN_{water}) depends on the transfer coefficient for the uptake of a VOC into a water body (k_{air_water}), the transfer area of the water body (A_{water}), and the difference in concentration between the VOC in greenhouse air ($[VOC]_{gr}$) and the VOC in the water body ($[VOC]_{water}$). This rate is described by Eq. (6.9).

$$SN_{water} = k_{air\_water} \cdot A_{water} \cdot ([VOC]_{gr} - [VOC]_{water}) \quad (6.9)$$

Assuming the above given sources and sinks to be the most dominant ones, the time course of the concentration of a VOC is described by Eq. (6.10) in which the individual sources and sinks constitute the mass-balance of the system.

$$V \cdot \frac{d[VOC]_{gr}}{dt} = SR_h + SR_B + SR_{air\_in} + SR_{mat} - SN_{air\_out} - SN_{mat} - SN_{water} - SN_{plant} - SN_{O3} \quad (6.10)$$

EXPERIMENTS

Experiment 1: introduction of VOCs together with air entering the greenhouse

Air samples were collected outside a small-scale greenhouse to estimate the entrance rate of VOCs by air entering this greenhouse. The method to derive the concentrations of VOCs in the ambient air outside of the greenhouse was based on active sampling and GC-MS analysis. This method has been described in detail by Jansen *et al.* (2009b) and provides detection limits in the order of 1 – 10 pptv. The concentrations of VOCs in the ambient air outside of

the greenhouse were assumed to be equal to the concentrations of VOCs in the air entering a greenhouse ($[VOC]_{air\_in}$). These concentrations were substituted into Eq. (6.3) to calculate the entrance rates of VOCs with air entering this greenhouse.

None of the VOCs considered in this study could be detected in the ambient air outside of the greenhouse given the detection limit of our system; that is approximately 1 pmol per mol of air for these VOCs (Jansen *et al.*, 2009b). The entrance of VOCs by air entering a greenhouse was therefore negligible as a sink and the term SR_{air_in} was neglected further on.

Experiment 2: removal of VOCs by air leaving the greenhouse

The removal of VOCs by air leaving a greenhouse depends on the volumetric flow rate of this air. The flow rate was determined for the small-scale greenhouse (Fig. 6.1 but without plants), using the tracer gas concentration decay technique (Bot, 1983). This technique is based on the decrease in concentration of a tracer gas inside and the nearly constant concentration of this tracer gas in the ambient air outside of the greenhouse. In this study, CO_2 was used as a tracer gas. The volumetric flow rate of air leaving the greenhouse (f_{air_out}) was calculated by Eq. (6.11). The tracer gas experiment was replicated three times.

$$[CO_2]_{gr}(t) = [CO_2]_{amb} + ([CO_2]_{gr,t=0} - [CO_2]_{amb}(t)) \cdot \exp(-\frac{f_{air\_out}}{V} t) \qquad (6.11)$$

The tracer gas experiment resulted in an exponential decrease of the tracer concentration inside the greenhouse. Based on Eq. (6.11) the flow rate of air leaving the greenhouse (f_{air_out}) was 0.041 ± 0.001 m^3 s^{-1}. This flow rate was substituted into Eq. (6.5) to calculate the removal of VOCs by air leaving the greenhouse.

Experiment 3: emissions of VOCs by greenhouse materials

Air samples were collected inside the greenhouse (Fig. 6.1 but without plants) to estimate whether the materials of this greenhouse emit any of the investigated VOCs. The method to derive the concentrations of VOCs inside of the empty greenhouse followed the procedure as described by Jansen *et al.* (2009b). This experiment was replicated three times.

None of the VOCs considered in this study could be detected in any of the samples obtained from the air inside the small-scale greenhouse without plants. The emission of VOCs by greenhouse materials was therefore neglected.

Experiment 4: adsorption/absorption of VOCs by greenhouse materials

The VOCs under investigation were evaporated in the small-scale greenhouse (Fig. 6.1) without plants to estimate whether greenhouse materials adsorb and/or absorb any of these VOCs. In order to evaporate these VOCs, we transferred about 10 mL of each VOC from GC-grade standards (Fluka, Milwaukee, WI, USA) into individual 25 mL glass vials which were immediately capped. The capped vials were then put in the centre of the greenhouse for at least 50 min to stabilise their temperature. After this period, the vials were uncapped to start evaporation. After 60 min, the vials were capped to end evaporation. Air samples were collected before vials were uncapped and 1, 2, 4, 7, 11, 16 and 24 hours after the vials were capped to determine the time courses of the concentrations of the evaporated VOCs. The method to derive these concentrations has been described in detail by Jansen *et al.* (2009b). The time courses of the concentrations of the evaporated VOCs were compared with the time course of the concentration of the CO_2 tracer gas to determine the adsorption/absorption rate of VOCs by greenhouse materials (SN_{mat}). This experiment was replicated three times with an interval of 48 h.

The time courses of the concentrations of all VOCs considered in this study were similar to the time course of the concentration of the CO_2 tracer gas. As an example, the time course of the concentration of (*Z*)-3-hexenol and the time course of the concentration of the CO_2 tracer gas are shown in Fig. 6.2.

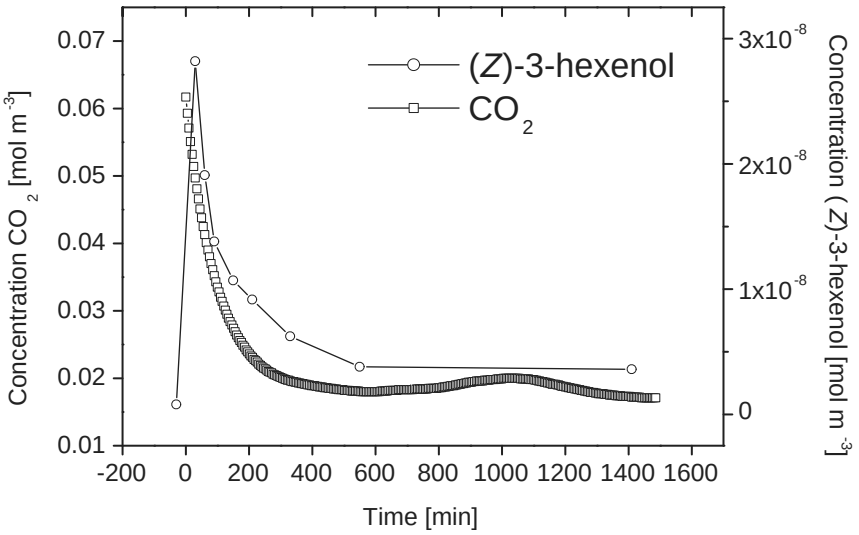

Fig. 6.2 Time course of the concentration of (*Z*)-3-hexenol and the time course of the concentration of the CO_2 tracer gas.

The similarity of the decay curves implies that in the greenhouse without plants, the VOCs were mainly removed by air going out of the greenhouse. Adsorption/absorption of VOCs by greenhouse materials was therefore neglected further on.

Experiment 5: adsorption/absorption of (Z)-3-hexenol by tomato plants

Tomato plants were only tested for adsorption and/or absorption of (*Z*)-3-hexenol since the other VOCs considered in this study are emitted from healthy tomato plants (see Jansen *et al.* (2009c) and references therein), which makes it impossible to distinguish between adsorption, absorption and emission. A laboratory set-up as described in detail by Heiden *et al*. (2003) (Fig. 6.3) was used to determine the adsorption/absorption rate of (*Z*)-3-hexenol by tomato plants.

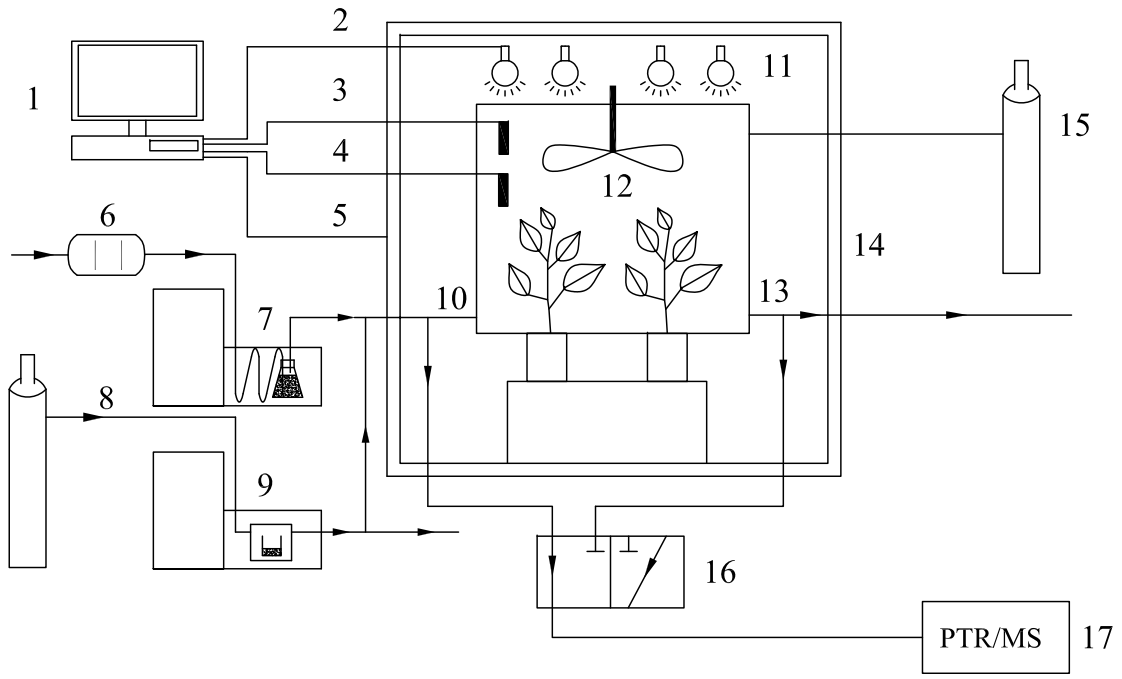

Fig. 6.3 Schematic illustration of the laboratory set-up used to determine the adsorptive/absorptive properties of tomato plants: (1) personal computer, (2) light control, (3) light intensity sensor, (4) dew point sensor and (5) temperature sensor, (6) air filters, (7) humidifier, (8) nitrogen supply, (9) set-up to add (*Z*)-3-hexenol into the chamber, (10) air entering the chamber, (11) lights, (12) Teflon fan, (13) air leaving the chamber, (14) temperature controlled housing, (15) CO_2 supply, (16) valve, and (17) proton transfer reaction – mass spectrometer (PTR/MS). This figure was modified after Jansen *et al.* (2009c).

The tomato plants used for this experiment were six or eight weeks old. Briefly, the shoot part of such a plant was enclosed in a 1.1 m^3 glass chamber. The light intensity on top of the plant was set at a photosynthetic photon flux density (PPFD) of 480 µmol photons m^{-2} s^{-1}. The temperature inside of the chamber was set at 20°C, the humidity inside of the chamber was set at 70% relative humidity and the CO_2 concentration inside of the chamber was maintained at 0.015 mol m^{-3} to mimic natural conditions. Clean and moistened air, supplemented with CO_2 was entering this chamber at a flow rate of 30 L min^{-1}. Approximately 5 min after enclosure of the shoot part, about 1.1×10^{-10} mol s^{-1} of (*Z*)-3-hexenol was added into this incoming air. A Proton Transfer Reaction – Mass Spectrometer (PTR/MS) was used to alternatively measure the concentration of (*Z*)-3-hexenol in the air entering and the concentration of (*Z*)-3-hexenol in the air leaving the chamber (measurements at m/z = 83, (Fall *et al.*, 1999)). This experiment was replicated twice with other individuals of tomato plants.

In none of these experiments, significant differences were found between the concentration of (*Z*)-3-hexenol in the air entering and the concentration of (*Z*)-3-hexenol in the air leaving the chamber (data not shown). The adsorption/absorption of (*Z*)-3-hexenol by tomato plants was therefore neglected.

Experiment 6: gas-phase reactions of VOCs with O_3

Colorimetric detector tubes (Kitagawa, Japan) were used to measure the concentration of O_3 in the air inside the small-scale greenhouse (Fig. 6.1). At the time of measurement, the greenhouse contained 60 tomato plants of about 1.8 m in height. The concentration of O_3 was measured in two independent replicates. For each replicate, 4.8 L of air was sucked through the tube.

In both experiments, the concentration of O_3 inside of the small-scale greenhouse including 60 tomato plants was below the detection limit of the method (< 3 nmol mol^{-1} air). Therefore, gas-phase reactions of VOCs with O_3 (SN_{O3}) were neglected.

Experiment 7: the transfer of VOCs into water bodies

Both the transfer area of water bodies as well as the transfer coefficient are neither predictable by simple theories nor determinable by straightforward measurements since both depend on a complex array of hydrodynamic and aerodynamic factors. We therefore conducted an experiment with plants being in the greenhouse to simulate realistic conditions. These plants served as source of water as a result of condensation of transpired water onto cold surfaces. Hence, in this experiment, an overall loss was measured:

$$\Sigma\text{Loss} = SN_{air\_out} + SN_{mat} + SN_{water} + SN_{plant} + SN_{O3} \quad (6.12)$$

The results described before indicated that greenhouse materials, tomato plants, and gas phase reactions are negligible sinks for VOCs and therefore, Eq. (6.12) reduces to Eq. (6.13):

$$\Sigma\text{Loss} = SN_{air\_out} + SN_{water} \quad (6.13)$$

To estimate the losses described in Eq. (6.13), we evaporated the VOCs considered in this study inside the greenhouse including 60 tomato plants of about 1.8 m in length. The evaporation of VOCs, the sampling of air, and the analyses of samples followed the procedure as described in Experiment 4. Assuming that the loss of the VOC by solution in the water bodies is proportional to the concentration of the VOC in the greenhouse air, this loss process is of first order. As the loss by removal with the air leaving the greenhouse is also a first order process, the decay processes can be described by Eq. (6.14).

$$[\mathrm{VOC}]_{gr}(t) = ([\mathrm{VOC}]_{gr,t=0}) \cdot \exp\left[\frac{f_{airout} + (k_{air\_water} \cdot A_{water})}{V} t\right] \qquad (6.14)$$

The decrease in concentration after evaporation was fitted to an exponential decay curve to calculate $k_{air\_water} \cdot A_{water}$.

Two different tomato crops were used to determine $k_{air\_water} \cdot A_{water}$ per VOC. Crop *A* was planted in February 2008 and crop *B* was planted in August 2008. For crop *A*, the VOCs were evaporated one time at 72, a second time at 74, and a third time at 76 days after planting. For crop *B,* the VOCs were evaporated one time at 76, and a second time at 78 days after planting.

Fig. 6.4 provides an example of the time courses of the concentrations of (*Z*)-3-hexenol, *β*-caryophyllene and methyl salicylate after vials were capped to stop evaporation.

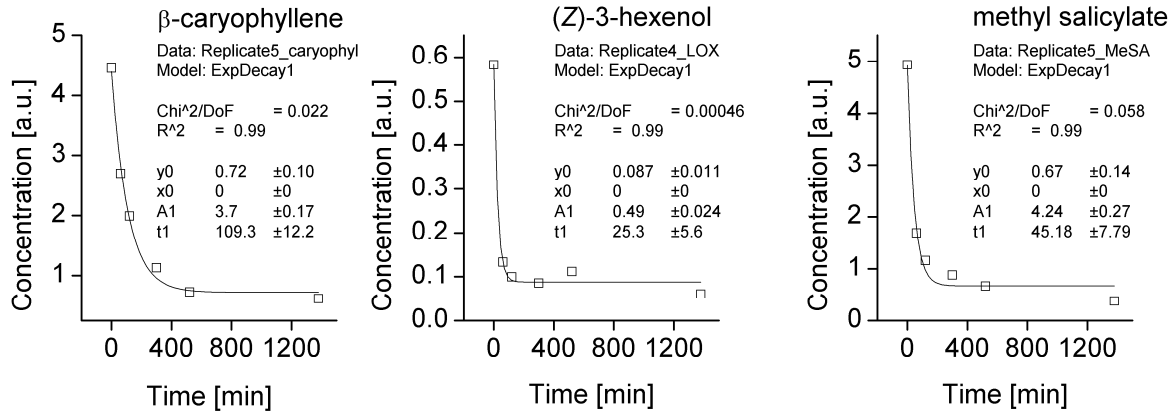

Fig. 6.4 Time courses of the concentrations of *β*-caryophyllene, (*Z*)-3-hexenol, and methyl salicylate after vials were capped to stop evaporation.

The results obtained in the different experiments are listed in Table 6.1.

Table 6.1 Calculated $k_{air\_water} \cdot A_{water}$ values for Crop *A* and Crop *B*.

	$k_{air\_water} \cdot A_{water}$ [$m^3\ s^{-1}$]					**Average (sd)**
Compound	**Crop *A***			**Crop *B***		
(*Z*)-3-hexenol	0.41	0.41	0.15	0.13	0.14	0.25 (0.15)
α-pinene[a]	0.00	0.01	0.02	0.00	0.03	0.01 (0.01)
α-terpinene	0.07	0.06	0.05	0.04	0.06	0.06 (0.01)
β-caryophyllene	0.02	0.02	0.02	0.00	0.02	0.01 (0.01)
methyl salicylate	0.04	0.05	0.04	0.06	0.06	0.05 (0.01)

[a] In two out of five replicates, the concentration of *α*-pinene at 1 h after evaporation was below the concentration of *α*-pinene at 2 h after evaporation. This phenomena was attributed to breakthrough which means that a compound is no longer retained on the sorbent. This seems plausible for *α*-pinene since it has a low breakthrough volume compared to other monoterpenes (Simon *et al.*, 1995). These two measurement points were therefore excluded when calculating the $k_{air\_water} \cdot A_{water}$ of *α*-pinene.

MODEL PREDICTIONS FOR LARGE-SCALE GREENHOUSES

Based on the results of experiments 1-7, Eq. (6.10) was reduced to:

$$V \cdot \frac{d[VOC]_{gr}}{dt} = SR_h + SR_B - SN_{air\_out} - SN_{water} \qquad (6.15)$$

This Eq. (6.15) was implemented in Matlab^{tm} (Release 14; The MathWorks Inc., MA, USA) to predict the effects of parameter changes on the concentrations of VOCs in a large scale greenhouse under four different scenarios. The main quantities determining these concentrations are the volume of the greenhouse, the emission flux densities of the individual VOCs, the total number of plants, the fraction of *B. cinerea* infected plants, the leaf area per plant, and the exchange of air with the environment.

Emission flux densities of VOCs by healthy and infected plants are required to calculate their emission rates. Table 6.2 provides the emission flux densities used in this study. These data were taken from our previous work in which tomato plants were severely infected with *B. cinerea* (Jansen *et al.*, 2009c).

Table 6.2 Emission flux densities of the volatile organic compounds used in this study. Data were taken from our previous work in which tomato plants were severely infected with *Botrytis cinerea* (Jansen *et al.*, 2009c, Jansen *et al.*, 2009d).

	Emission flux density [nmol m^{-2} s^{-1}]	
Compound	**Healthy**	***Botrytis cinerea* infected**
(Z)-3-hexenol	0	3.1×10^{1}
α-pinene	2.1×10^{-2}	3.0×10^{-2}
α-terpinene	2.5×10^{-2}	3.9×10^{-2}
β-caryophyllene	1.1×10^{-3}	1.9×10^{-3}
methyl salicylate	1.1×10^{-1}	3.2×10^{-1}

Four different scenarios were considered in this study. Table 6.3 presents the volume of the greenhouse, the total number of tomato plants, the proportion of *B. cinerea* infected tomato plants, the leaf area per plant, and the flow rate of air leaving the greenhouse for these four scenarios. Table 6.3 also presents the excitation period. This period of time represents the time in which the *B. cinerea*-induced increase in emission of VOCs from a certain proportion of plants is above the baseline level emission of healthy plants.

Table 6.3 Volume of the greenhouse (V), total number of tomato plants, proportion of *Botrytis cinerea* infected tomato plants, the excitation period, the leaf area per plant (A_{plant}), and the flow rate of air leaving the greenhouse (f_{air_out}) for scenario 1, 2, 3, and 4.

	Scenarios			
	1	2	3	4
Volume [m^3] [a]	5×10^4	5×10^4	5×10^4	5×10^4
Total number of plants [-] [b]	2.2×10^4	2.2×10^4	2.2×10^4	2.2×10^4
Botrytis cinerea infected plants [%]	0.5	5	0.5	5
Excitation period [min]	60	60	60	60
Leaf area per plant [m^2]	1	1	1	1
Flow rate of air leaving the greenhouse [$m^3\ s^{-1}$]	4.6 [c]	4.6 [c]	278 [d]	278 [d]

[a] Volume is a based on a floor area of 10^4 m^2 and a height of the greenhouse of 5 m; these dimensions are common for Dutch greenhouses.

[b] Total number of tomato plants reasonable for the corresponding greenhouse volume and floor area.

[c] This flow rate is one-third of the corresponding greenhouse volume per hour; a reasonable value for modern Dutch greenhouses when windows are closed.

[d] This flow rate is 20-times the corresponding greenhouse volume per hour; a reasonable value for modern Dutch greenhouses when windows are fully opened.

Eq. (6.15) was used to predict the baseline and *B. cinerea*-induced concentrations of VOCs in the greenhouse atmosphere under the four different scenarios as listed in Table 6.3. For these scenarios, the emissions from the healthy plants (SR_h) led to an increase of VOC concentrations until a steady state concentration was reached. These steady state concentrations were determined by the sinks and sources given in Eq. (6.15) with $SR_B = 0$. After steady state was reached, it was assumed that a given proportion of plants (Table 6.3) became infected by *B. cinerea*: in this case all at the same time, t = 1500 min. As a consequence, the infected plants emit pulses of VOCs. This led to a temporary increase in VOC concentrations in the greenhouse air which in height depend on the given scenario. As an example, in Fig. 6.5 we show the predicted concentrations of methyl salicylate under scenario 1, 2, 3, and 4.

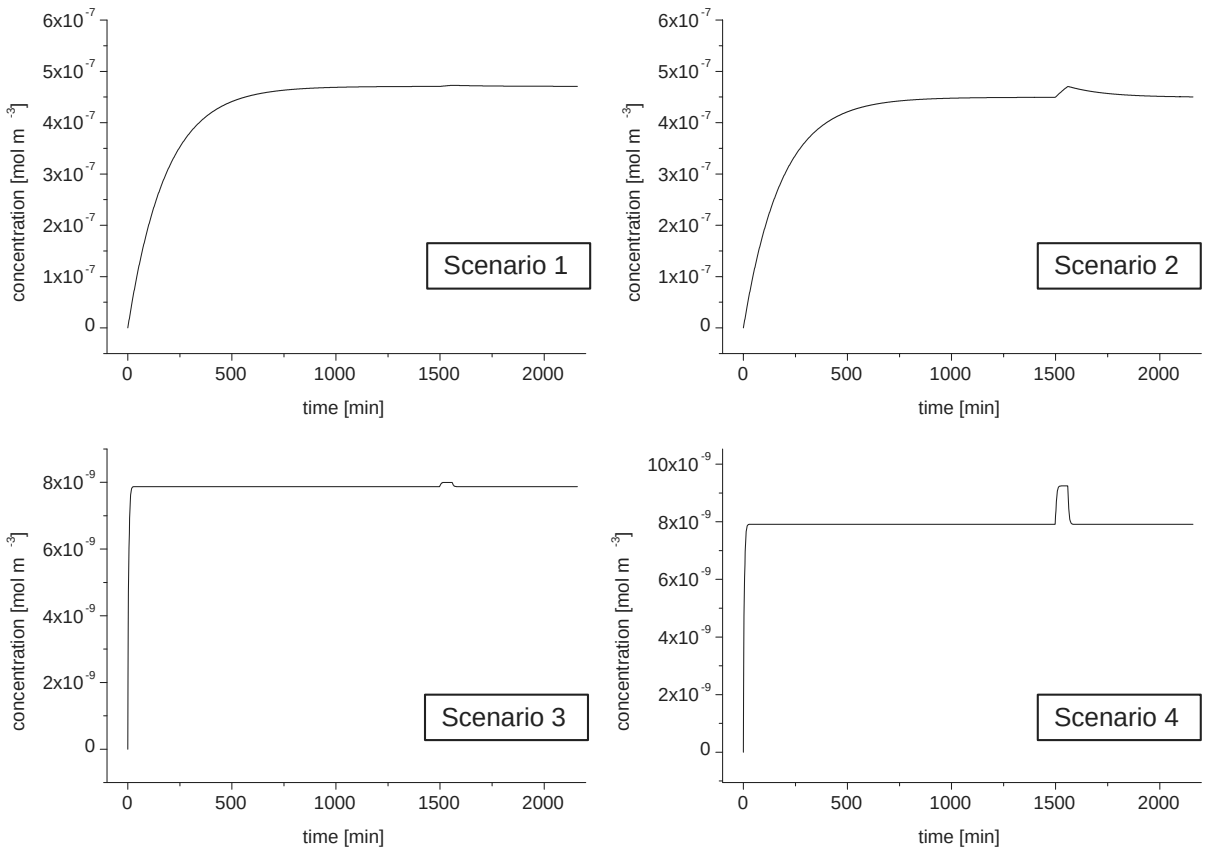

Fig. 6.5 The predicted baseline and *Botrytis cinerea*-induced concentrations of methyl salicylate under scenario 1, 2, 3, and 4. The scenarios are presented in Table 6.3. At t = 0 min, healthy plants are introduced into the greenhouse. At t = 1500 min, a pulse type increase with an excitation period of 60 min was simulated to imitate a *B. cinerea* infection.

Table 6.4 provides an overview of the predicted baseline and *B. cinerea*-induced increase in concentrations of the VOCs used in this study (Table 6.2).

Table 6.4 Predicted baseline and *Botrytis cinerea*-induced increase in concentration of the volatile organic compounds (*Z*)-3-hexenol, *α*-pinene, *α*-terpinene, *β*-caryophyllene, and methyl salicylate under four different scenarios. The scenarios are presented in Table 6.3.

	Compound				
	(*Z*)-3-hexenol	*α*-pinene	*α*-terpinene	*β*-caryophyllene	methyl salicylate
Scenario 1					
Baseline[1]	0	9.995E-08	1.177E-7	5.235E-9	4.719E-7
Induced[1]	2.030E-7	1.003E-07	1.181E-7	5.254E-9	4.745E-7
Increase[2]	∞	0.35	0.34	0.36	0.55
Scenario 2					
Baseline[1]	0	9.995E-08	1.177E-7	5.235E-9	4.719E-7
Induced[1]	2.030E-6	1.008E-07	1.189E-7	5.301E-9	4.875E-7
Increase[2]	∞	0.85	1.02	1.26	3.31
Scenario 3					
Baseline[1]	0	1.662E-09	1.978E-9	8.705E-11	7.912E-9
Induced[1]	1.226E-8	1.665E-09	1.984E-9	8.736E-11	7.999E-9
Increase[2]	∞	0.18	0.30	0.36	1.10
Scenario 4					
Baseline[1]	0	1.662E-09	1.978E-9	8.705E-11	7.912E-9
Induced[1]	1.226E-7	1.697E-09	2.033E-9	9.021E-11	8.783E-9
Increase[2]	∞	2.11	2.78	3.63	11.01

[1] Concentration in mol m^{-3}

[2] Increase in %

DISCUSSION

Detection of *B. cinerea* infections through plant-emitted VOCs requires analytical instruments with appropriate precision, detection limits and time resolution. The VOCs considered in this study have been measured routinely with precision values expressed as relative standard deviation (RSD) of 10%, detection limits of 1 nmol m^{-3}, and a time resolution of 1 h. For instance, Greenberg *et al.* (1994) used an instrument that consisted of cryo-trapping and gas chromatography - flame ionization detection (GC-FID) to detect VOCs with a precision of ca. 10% RSD at detection limits of less than 1 nmol m^{-3}. Similar precision and detection limits

have been achieved using sorbent-trapping and GC-MS (Karl *et al.*, 2008, Vercammen, 2002). The precision and detection limits of these two analytical instruments were compared with the *B. cinerea*-induced increase in concentration of (*Z*)-3-hexenol, *α*-pinene, *α*-terpinene, *β*-caryophyllene, and methyl salicylate as given in Table 6.4, to determine whether the increase is detectable with these instruments.

The precision and detection limits of GC-MS or GC-FID are low enough to detect the *B. cinerea*-induced increase in concentration of (*Z*)-3-hexenol under all scenarios. For these scenarios, the relative increases in concentration of *α*-pinene, *α*-terpinene and *β*-caryophyllene are below the precision of GC-MS or GC-FID to be detectable. The detection limit of these instruments are insufficient to detect *β*-caryophyllene under scenario 3 and 4, *i.e.* windows fully opened. The *B. cinerea*-induced increase in concentration of methyl salicylate is only detectable under scenario 4, *i.e.* 5% of the plants infected and windows fully opened to prevent that fluctuations in the concentrations of methyl salicylate are levelled out. It should be mentioned that the relative increase in concentration of methyl salicylate under scenario 4 is not simple to detect since this increase is near to the RSD of GC-MS and GC-FID. The estimates of the detectability of *B. cinerea*-induced increase in concentration of (*Z*)-3-hexenol, *α*-pinene, *α*-terpinene, *β*-caryophyllene, and methyl salicylate are summarised in Table 6.5.

Table 6.5 Detectability of the *Botrytis cinerea*-induced increase in concentration of (*Z*)-3-hexenol, *α*-pinene, *α*-terpinene, *β*-caryophyllene, and methyl salicylate under scenario 1, 2, 3 and 4. An instrument with a precision of 10% relative standard deviation and a detection limit of 1 nmol m^{-3} is used as a reference. The increase in concentration of the compounds are presented in Table 6.4. Scenarios are presented in Table 6.3.

	Detectability (precision / detection limit)			
Compound	Scenario 1	Scenario 2	Scenario 3	Scenario 4
(*Z*)-3-hexenol	+ / +	+ / +	+ / +	+ / +
α-pinene	- / +	- / +	- / +	- / +
α-terpinene	- / +	- / +	- / +	- / +
β-caryophyllene	- / +	- / +	- / -	- / -
methyl salicylate	- / +	- / +	- / +	+ / +

The results in Table 6.5 are bases on the momentaneous concentrations of VOCs. In practice, VOCs are often pre-concentrated to achieve the detection limits of commonly applied analytical instruments. The period of time required for pre-concentration depends on the concentration of the VOCs of interest in the air. A generally accepted value is 60 minutes at concentrations in the order of nmol m^{-3}. Also the separation of VOCs in the mixture requires a certain amount of time in the order of 15-45 min. A sensitivity analysis should include the separation and pre-concentration periods to determine the detectability of *B. cinerea*-induced increase in concentrations of VOCs in large-scale greenhouses. Such analysis should also include the period of time in which the *B. cinerea*-induced increase in emission of VOCs from a certain proportion of plants is above the baseline level emission of healthy plants. For instance, the *B. cinerea*-induced increase in concentration of methyl salicylate is also detectable under scenario 2, *i.e.* 5% of the plants infected and windows closed if this period of time increases from 60 to at least 360 minutes (data not shown). Furthermore, a sensitivity analysis should include the emission flux densities of methyl salicylate by healthy tomato plants. These values were obtained under laboratory conditions. It is doubtful whether laboratory conditions are suitable to determine methyl salicylate emissions from healthy plants since stress due to enclosure of tomato plants -a prerequisite for analysing plant emissions at the laboratory scale- also led to increased emissions of methyl salicylate (Ament, 2006).

The ambient air nearby a greenhouse was analysed to explore the importance of incoming air as source of plant VOCs inside a greenhouse. The absence, or at least very low concentrations of these VOCs in ambient air, was counter-intuitive since nearby vegetation such as trees, hedges, and field crops are potential sources of VOCs (Army *et al.*, 1991), especially upon harvest (*e.g.* Davison *et al.*, 2008). We assume that the amount of vegetation present near the greenhouse was insufficient to induce considerable emissions of VOCs. Also crops inside nearby located greenhouses are possible sources of VOCs. But, the number and size of nearby greenhouses was probably too small to play an important role. However, these sources remain relevant for large-scale greenhouse because they are, in general, surrounded by many other large-scale greenhouses. The absence of relevant VOCs may also be explained by gas-phase reactions in the troposphere in which VOCs are degraded. Such degradation seems plausible since the VOCs used in this study have tropospheric lifetimes in the order of minutes to hours (Atkinson and Arey, 2003, Canosa-Mas *et al.*, 2002). The tropospheric lifetime, together with

wind direction and wind speed are important characteristics to consider since they determine the distance VOCs can move before degradation. In future studies, the air surrounding such large-scale greenhouses should be analysed to give an impression about VOC concentrations in this air. This should preferably be done at different times of the year and different times of the day due to the seasonal and diurnal rhythm of VOC emissions by vegetation (*e.g.* Tarvainen *et al.*, 2005).

The air in a greenhouse without plants was analysed to determine whether greenhouse materials emit any of the investigated VOCs. The materials of the small-scale greenhouse did not emit substantial amounts of these VOCs. To analyse the emission of VOCs from most common greenhouse materials seems the preferred method to find out whether these materials emit significant amounts of relevant VOCs. However, glass is one of the main materials of a modern greenhouse and since glass emit almost no VOCs, this source process is more or less irrelevant.

A tracer gas was used to determine the removal rate of VOCs by air leaving a greenhouse. This turned out to be the most dominant sink for α-pinene, α-terpinene and β-caryophyllene. Whether or not windows are opened will have a large effect on this removal rate. The windows of a large scale greenhouse are often closed, but also regularly opened to dehumidify the greenhouse, to cool the greenhouse, and to supply extra CO_2. Fluctuations in VOC concentrations caused by closing and opening of windows may then be misinterpreted. The effect of this procedure should be studied in more detail to find out whether this may obstructs the interpretation of plant VOC concentrations. Trials are currently ongoing to test the effect of different window configurations on VOC concentrations in greenhouse air.

VOCs were evaporated inside a greenhouse without plants to investigate whether greenhouse materials adsorb and/or absorb large amounts of VOCs. The materials of the small-scale greenhouse did not adsorbed/absorbed substantial amounts of relevant VOCs. But, these processes should not be neglected completely since many researchers have demonstrated that materials have the capacity to absorb and/or adsorb VOCs (Jorgenson, 1999, Singer *et al.*, 2004). However, glass is one of the main materials of a modern greenhouse. Most chemicals hardly absorb and/or adsorb onto glass materials which makes this sink process not so relevant.

VOCs were evaporated inside a greenhouse including plants to determine the extent to which VOCs might be removed due to transfer into water bodies. The results obtained in this experiment suggested that this process is an important sink strength for the water soluble VOCs (*Z*)-3-hexenol and methyl salicylate. These two VOCs are possible indicators for *B. cinerea* infection of tomato (see Table 6.2). It is therefore of utmost importance to study this sink in more detail to determine whether or not these VOCs are detectable under conditions that occur in large scale greenhouse.

Well defined amounts of (*Z*)-3-hexenol were supplied to the air surrounding the shoots of tomato plants to study whether they adsorb and/or absorb this VOC. Shoots of tomato plants did not adsorbed/absorbed substantial amounts of (*Z*)-3-hexenol. However, other plant species may absorb and/or absorb VOCs since this process might depend on the plant species considered as suggested by Böhme *et al.* (1999). Unfortunately, based on the experiments we made, we cannot exclude that tomato plants adsorb and/or absorb any of the other VOCs considered in this study.

Colorimetric tubes were used to determine O_3 concentrations in the greenhouse, and, as expected no ozone was detectable. It is long known that plants take up ozone very efficient (*e.g.* Neubert *et al.*, 1993) and thus, concentrations of ozone in a greenhouse with high plant density should be very low. Therefore, gas phase reactions of plant emitted VOCs with O_3 inside greenhouse will be negligible as sink. Other tropospheric oxidants as OH and NO_3 are produced from O_3. Thus, without ozone in the greenhouse air there is also no production of such oxidants. Furthermore the lifetime of these compounds is in the range of seconds and an introduction with air entering the greenhouse will not lead to accumulation of such compounds making also these oxidants unimportant. In summary, we believe that gas phase reactions can be ruled out as a sink for the emitted VOCs in greenhouses with high plant density.

In the present study, four sources were regarded as relevant for the presence of VOCs in greenhouse air. An additional source that should be considered is plant debris such as excised shoots after pruning. Such debris is often present in a greenhouse and may then result in increases in VOC concentrations due to their emissions.

A major issue with respect to the implementation of VOC based crop monitoring is the spatial distribution of VOCs inside a large-scale greenhouse. Our model predictions for large-scale greenhouses relied on the assumption that the air in the greenhouse is perfectly mixed; an assumption often made in classical greenhouse studies (Roy *et al.*, 2002). However, VOC concentrations will probably show spatial variation since the air in a greenhouse is never perfectly mixed. These variations are influenced by the characteristics of a greenhouse, such as the ventilation system and the temperature distribution. The local concentrations can show a high variability thus leading to lower or higher concentrations than the ones predicted in this study. Furthermore, *B. cinerea* infections are likely to occur in patches. This will also affect the spatial distribution of VOCs and result in an increase in local concentrations. Currently, computational fluid dynamics (CFD) has been acknowledged as an appropriate tool to calculate airflow distributions in greenhouses (*e.g.* Campen and Bot, 2003). CFD may also be useful to predict airflow distributions and concentrations of VOCs in greenhouse air which then might help to predict the best locations for air sampling.

In the present work, we ignored the effect of light intensity and leaf temperature on VOC emissions by plants. However, light intensity and leaf temperature affect methyl salicylate and terpenoid emissions from tomato (Farag and Paré, 2002, Vercammen *et al.*, 2001) which may then superimpose possible *B. cinerea* induced concentration differences. This effect may have caused the absence of any significant increase in terpenoid concentration after *B. cinerea* infection (Jansen *et al.*, 2009a). However, the increase in emissions of methyl salicylate as a result of *B. cinerea* seems much stronger then the effect of common temperature and light fluctuation in a greenhouse (Jansen *et al.*, 2009a). For (*Z*)-3-hexenol, the effect of light and temperature is unimportant since unstressed tomato plants do not emit this VOC.

The present study was based on tomato and the infection of this plant species with *Botrytis cinerea*. The motivation to select tomato and *B. cinerea* as our model organisms was twofold. First, tomato is the major greenhouse crop in most parts of the world, which includes Northern Europe, (Heuvelink, 1995). Second, *B. cinerea* remains the major constraint for greenhouse tomato production worldwide (*e.g.* Eden *et al.*, 1996, Elad and Stewart, 2004). However, plant emitted VOCs may also be used to monitor other vegetable- or ornamental crop species for pathogen infections and to monitor crops for the presence of other crop health

problems. This is in accordance with the enormous amount of laboratory studies in which increased emissions of VOCs have been reported upon decline of plants' health (Kesselmeier and Staudt, 1999, Peñuelas and Llusià, 2001).

CONCLUSION

Based on model predictions, the *B. cinerea*-induced increase in concentration of the volatile plant hormone methyl salicylate is detectable in a large-scale tomato production greenhouse when at least three conditions are met: (a) windows are fully opened, and (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 1 h, and (c) 5% of the plants are infected. The *B. cinerea*-induced increase in concentration of methyl salicylate is also detectable when (a) windows are closed, and (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 6 h, and (c) 5% of the plants are infected.

The increase in concentration of the lipoxygenase product (*Z*)-3-hexenol is detectable under all scenarios. However, it is expected that besides infected plants, many additional sources of lipoxygenase products exist including plant debris and nearby field crops especially upon harvest or stress. Plant debris is nearly always present in greenhouses, and harvest and/or stress of nearby crops is extremely difficult to predict. The *B. cinerea*-induced increases in concentration of the three trichome damage related VOCs *α*-pinene, *α*-terpinene and *β*-caryophyllene are probably undetectable in a large-scale tomato production greenhouse. Therefore, it is recommended to focus on the detection of methyl salicylate to indicate *B. cinerea* infections in large-scale tomato production greenhouses.

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CHAPTER 7

An automated method for signal processing of GC-MS data

INTRODUCTION

Regular human inspections are still the primary method by which greenhouse managers assess the health status of their crops. These human inspections are indispensable. However, technological developments might help to detect emerging health problems at an early stage which will make it easier to manage and control them. A novel approach to support the inspection of greenhouse crops is based on the measurement of volatile organic compounds (VOCs) emitted by unhealthy plants. This approach has attracted some serious interest over the last decade.

In pursuit of this interest, studies were undertaken at the laboratory-scale to pinpoint marker VOCs that can be used to indicate health problems of tomato and cucumber (Laothawornkitkul *et al.*, 2008, Thelen *et al.*, 2006). In addition to these laboratory studies, pilot studies were performed in order to verify the validity of these marker VOCs under real-world conditions (Karl *et al.*, 2008, Markom *et al.*, 2009).

Different measurement techniques have been employed to study health aspects of agricultural plant species based on VOCs. A great deal of these studies employed electronic noses (Baratto *et al.*, 2005) but also biosensors were employed (Schütz *et al.*, 1995). However, in most of these studies, the analysis of VOCs was accomplished by a combination of gas chromatography (GC) for separation and mass spectrometry (MS) for detection and identification of the specific VOCs (Jansen *et al.*, 2009c). The popularity of GC-MS is based on a favourable combination of high selectivity and resolution, good accuracy and precision, wide dynamic concentration range, and high sensitivity (Santos and Galceran, 2002). GC-MS is therefore considered as a serious candidate for health monitoring through analysis of air inside high-input greenhouse facilities. Conventional GC-MS systems are not suitable for that since these are delicate instruments usually restricted to laboratory use. As a consequence, air samples collected in the greenhouse should be transferred to the laboratory for further analysis. However, this transfer of samples introduces a time delay which is undesirable in case the detection of health problems require an immediate act, *e.g.* in case of the detection of a highly transmittable disease. Air samples should therefore preferably be analysed on-site. More robust GC-MS systems have therefore appeared on the market and have been applied, for example, to detect air contaminants in field settings (Eckenrode, 2001, Smith *et al.*, 2005)

and to monitor a biogas tower reactor for the presence of potentially toxic VOCs (Matz *et al.*, 1998).

A widely recognized difficulty associated with GC-MS application is the large and complex data generated by this instrument. As a consequence, experienced analysts are often required to process this data in order to depict the concentrations of the chemical compounds of interest (Malmquist *et al.*, 2007). Such manual processing would typically incur high costs associated with labour. This aspect was identified as a limiting factor for the effective application of GC-MS based crop health monitoring in the 21st century. However, developments in computer science technology and software have increased the opportunity to automatically process GC-MS data at an affordable price.

Numerous software packages are developed for the automatic extraction of relevant information from complex GC-MS data (reviewed by Katajamaa and Orešič, 2007). The algorithms implemented in these software packages rely on digital filters and univariate statistics for data smoothing, noise reduction, and baseline correction (Li *et al.*, 2002). Additional alignment algorithms are often implemented to correct for chromatographic peak shifts (Skov *et al.*, 2007). The majority of these software packages have their roots in metabolomics: ”the study of the unique chemical fingerprints that specific cellular processes leave behind" (Daviss, 2005). Often, these software packages are then successfully applied to find novel compounds that explain differences between large series of mass spectrometric data (Jonsson *et al.*, 2004). However, it is still unknown whether these algorithms are also useful to automatically extract signals that represent health associated VOCs in order to determine the concentrations of them. In this study, the processing algorithms implemented in the MetAlign^{TM} software package were validated for that. Thus, the objective of this study was to assess whether or not GC-MS data can also be automatically processed in order to determine the concentrations of crop health associated VOCs.

MATERIALS AND METHODS

Experimental datasets

The experimental dataset employed in this study was acquired from the chemical analysis of air samples collected in a small-scale greenhouse. Throughout a six weeks growing period,

the air inside this greenhouse was sampled directly before and just after artificial damage of a tomato crop. This artificial damage was imposed to the plants on a weekly interval and was supposed to simulate plant damage similar to that caused by plant health issues such as herbivore infestation or pathogen infection. The analysis of the air samples were performed offline using a gas chromatograph coupled to a mass spectrometer (GC-MS). The simplest data output from the mass spectrometer analyzer is a measurement of the total ion current strength (TIC) versus time. This is basically a chromatographic output representing a summation of the signal strength of all the ions produced by the mass spectrometer at a given time. Two typical examples of such chromatographic output obtained before and after damage of the tomato plants are presented in Fig. 7.1.

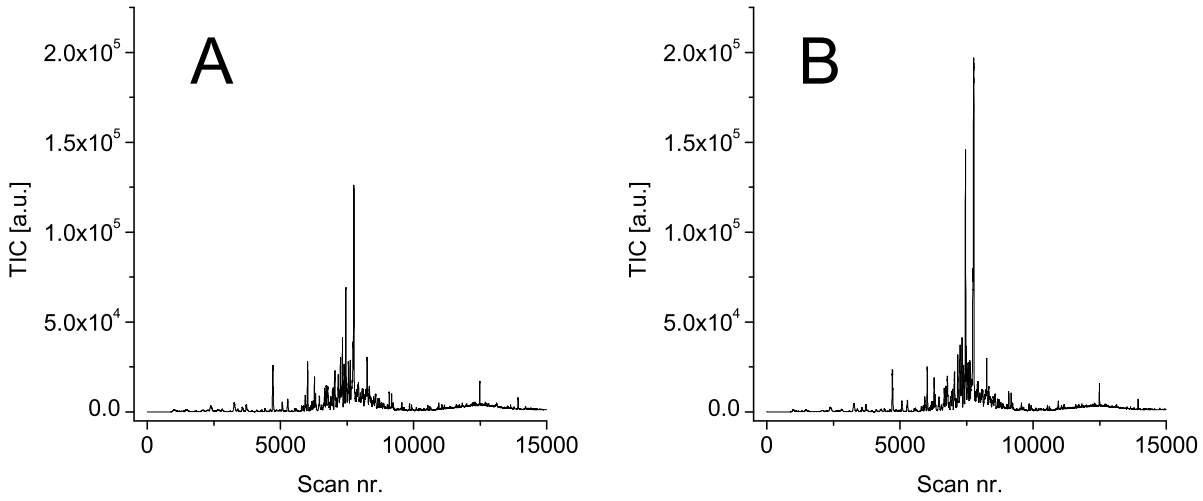

Fig. 7.1 Typical chromatographic profiles obtained from analysing the air in a greenhouse. Data were obtained in week nr. 6; before (A), and directly after damage of tomato plants (B). (TIC = total ion current)

The actual data output content is much more complex since the data block produced is three dimensional; TIC versus time versus mass-to-charge ratios (m/z); more details can be found in McMaster (2008). A graphical way to present the three dimensional structure of GC-MS data is provided in Fig. 7.2.

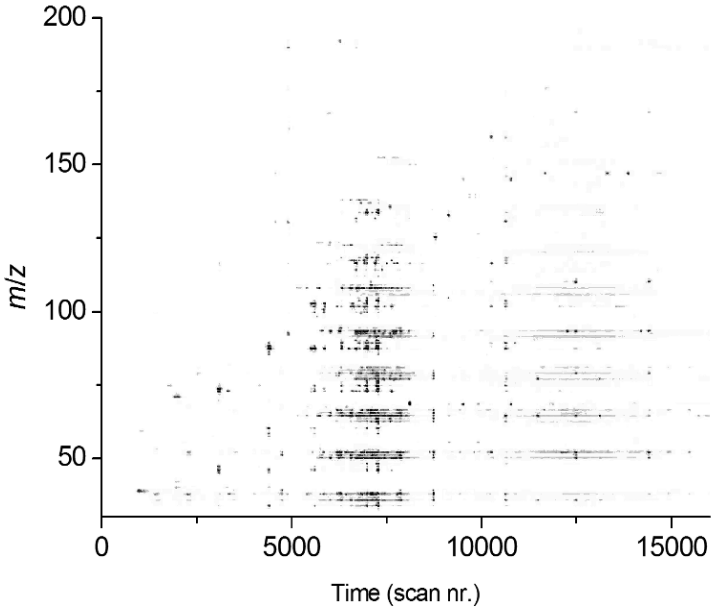

Fig. 7.2 Three dimensional gas chromatography – mass spectrometry data display. Data were obtained in week nr. 6 before damage of tomato plants. Light grey colours represent low intensities of the corresponding *m/z* values while dark grey colours represent high intensities of the corresponding *m/z* values. (*m/z* = mass-to-charge ratio)

The experimental equipment and the instrumental settings

The air samples were collected by purging 18 L of air from the greenhouse through stainless steel cartridges (Markes International Ltd, Lantrisant, UK) packed with 200 mg of Tenax-TA 20/35 (Grace-Alltech, Breda, The Netherlands). Air was purged through these cartridges at 300 mL min^{-1} for 60 min. The air samples were transferred to the laboratory for analysis. Before analysis, the cartridges were dry-purged with helium at ambient temperature with a flow of 100 mL min^{-1} for 10 min to remove water. Analytes were desorbed from the cartridges using thermal desorption at 250°C for 5 min under a flow of 30 mL min^{-1} of helium, and subsequently concentrated in an electronically-cooled focusing trap at -5°C (UltrA-TD™ and Unity™; Markes International Ltd). Analytes were then transferred to the column by heating the cold trap to 250°C at approximately 40°C s^{-1}. To prevent overloading of the analytical system, most samples were split prior to injection. Air samples obtained when plants were relatively small were analysed in splitless mode while samples obtained in case of large plants were analysed at split inlet modes between 1:6 and 1:24.

A gas chromatograph was used to separate the mixture of analytes (Trace GC UltrA^{TM}; Thermo Electron Corporation, Auston, TX, USA). The capillary column (Rtx-5 MS, 30 m × 0.25 mm internal diameter × 1 μm film thickness; Restek, Bellefonte, PA, USA) was held at the initial temperature of 40°C for 3.5 min followed by a linear gradient of 10°C min^{-1} to 280°C and a hold of 2.5 min resulting in an overall runtime of almost 33 min. The carrier gas was nitrogen of 99.999% purity and the column flow was approximately 1 mL min^{-1}.

The mass spectrometry was performed on a quadrupole mass spectrometer (Trace DSQ^{TM}; Thermo Electron Corporation). The mass scan range was set from 45 to 450 amu (atomic mass unit) at a scan rate of 5077 amu sec^{-1} and the electron ionization energy was set at 70 eV. The response of the mass spectrometer was assumed to be linear up to 2 × 10^8 ion counts per mass.

Manual processing of data

Manual processing of data was carried out by extracting the signals representing four VOCs: 2-carene, *α*-phellandrene, limonene, and *β*-phellandrene. These VOCs were purchased (Fluka, Buchs, Switzerland) and subsequently injected into the GC-MS to determine their scan numbers (retention time). The corresponding peaks in the total ion chromatogram were manually located at these scan numbers. The TIC areas underneath these peaks were manually integrated using an appropriate software package (XCalibur 2.0; Thermo-Finnigan, San Jose, CA, USA). This software package was also used to extract the corresponding peak areas in the selective ion chromatograms (SIC) using *m/z* 93 as characteristic fragment. The ratio between the TIC areas and SIC areas, and results from a calibration were used to quantify VOC concentrations. The calibration procedure itself has been described before by us (Jansen *et al.*, 2009b).

Automatic processing of data

The GC-MS data was automatically processed by the MetAlign^{TM} software package in which the following steps were carried out: (1) data smoothing by digital filters related to the average peak width, (2) estimation and storage of local noise as a function of retention time and mass peaks, (3) baseline correction of mass peaks and introduction of a threshold to realise noise reduction, (4) scaling, calculation and storage of peak maximum amplitudes, (5) between chromatogram alignment, (6) iterative fine alignment by including an increasing

number of mass peaks with lower signal-to-noise (S/N), significant difference filtering at user-defined significance thresholds and minimum x-fold ratios and (7) output of data back to the MS-platform.

To correct for the split levels used, data were scaled to the chemical compound naphthalene (*m/z* =128 at scan nr. 9520). Naphthalene was selected for scaling because this compound is not released from plants and was always present in almost constant concentration inside the greenhouse (Jansen *et al.*, 2008). Scaling to naphthalene was also used to correct for variability in GC-MS sensitivity, *e.g.* due to contamination of the ion source after cleaning the ion source. The quantification of VOC concentrations followed the procedure in Jansen *et al.* (2009b) corrected for MetAlign's peak area to intensity transformation. Parameters of MetAlign were set according to the specific scaling requirements and the chromatographic and mass spectrometric conditions used in the experiments (Table 7.1).

Table 7.1 MetAlign settings used to automatically process the GC-MS data.

Setting	**Value**
Retention begin (scan nr.)	0
Retention end (scan nr.)	15000
Maximum amplitude	200000000
Peak slope factor	0.5
Peak threshold factor	1
Average peak width at half height	20
Scaling	Marker peak
Nominal mass	128 at scan nr. 9520
Initial peak search criteria : maximum shift begin of 1st region	15
Initial peak search criteria : maximum shift end of 1st region	50
Maximum shift per 100 scans	35
Pre-align processing	Iterative
Minimum S/N ratio	10

RESULTS

The overall time needed to process the data manually was approximately 1 h. The results of the manual processing of data showed a weekly increase in concentrations of 2-carene, α-phellandrene, limonene, and β-phellandrene upon artificial damage.

The overall time needed to process the data automatically was approximately 10 min on a Pentium IV 1.5 GHz PC. Besides signals that represented the marker compounds, more than 3000 signals showed peak drift (Fig. 7.3). The differences in scans was especially large for highly volatile compounds which elute early (scan nr. < 2000) and for non-volatiles which elute late (scan nr. > 8000).

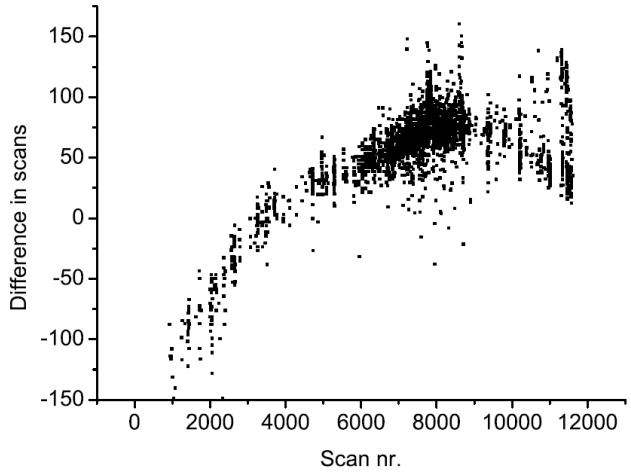

Fig. 7.3 Typical example of the differences in scans between two data-files.

Two data files were randomly selected to evaluate the implemented processing algorithms of MetAlign. These two data files showed significant difference in the scan numbers of the target compounds (Fig. 7.4). The phenomenon of drifted peaks and the effect of processing the data are illustrated in Fig. 7.4. This figure represents the effect of processing the two data files in a small part of the chromatogram (scan nr. 7500 – 8000). The figure is therefore not more than an impression of baseline correction, noise reduction, scaling and alignment.

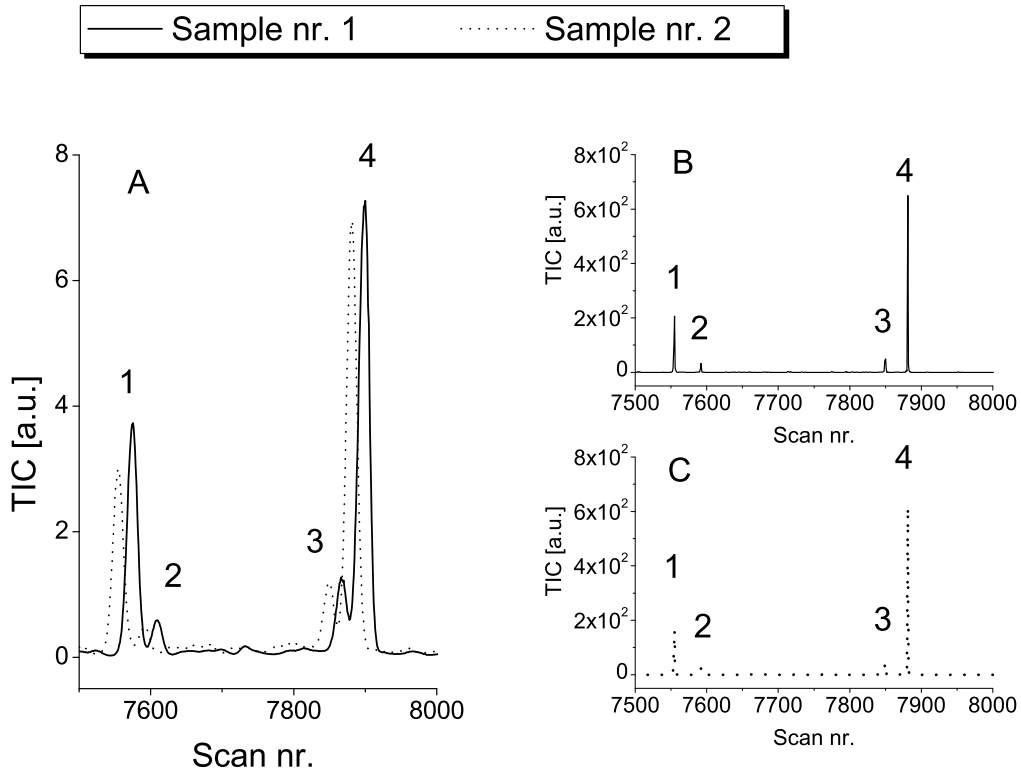

Fig. 7.4 Impression of data processing for signals that represent the concentration of (1) 2-carene, (2) *α*-phellandrene, (3) limonene, and (4) *β*-phellandrene. Provided are: (A) unprocessed data of sample nr.1 and nr. 2; (B) baseline corrected, scaled, noise reduced and aligned data of sample nr. 1; (C) baseline corrected, scaled, noise reduced and aligned data of sample nr. 2. TIC = total ion current

The results of automatic processing of the data resulted in concentrations similar to that after manual processing. The time course of the concentrations of *α*-pinene is provided in Fig. 7.5 to demonstrate this similarity.

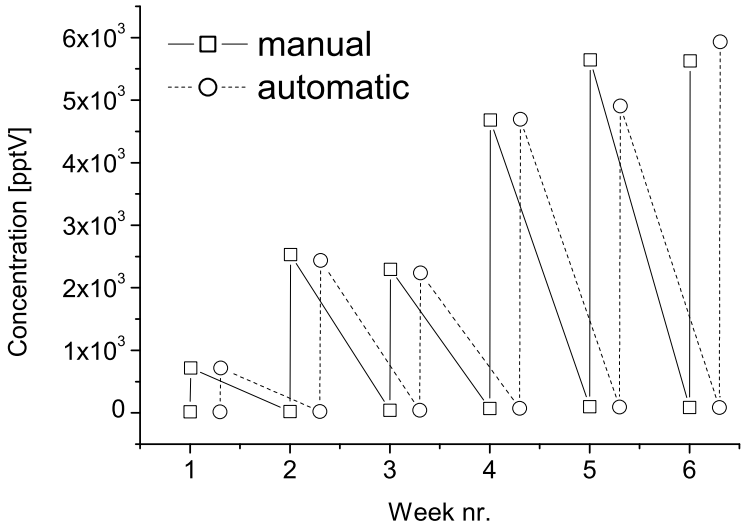

Fig. 7.5 Time course of the concentration of 2-carene after manual and automatic processing of gas chromatography – mass spectrometry data. The data points representing the automatically processed data have been offset to allow comparison.

DISCUSSION

The results of this study demonstrate that GC-MS data can be automatically processed in order to accurately determine the concentrations of health associated VOCs in a greenhouse. The processing of data was performed using MetAlign; a freeware software tool that has been effectively applied to process mass spectrometric data obtained from the quality control of fruits, plant-oil, drink-water, and grass (Lommen *et al.*, 2007, Tikunov *et al.*, 2005). This tool was also applied in the field of metabolomics which aims to develop and apply strategies for the global analysis of metabolites in cells, tissues and fluids (de Vos *et al.*, 2007). This study demonstrates how knowledge obtained from that rapidly expanding field can be used in an agricultural engineering setting.

An important disadvantage of MetAlign is that the algorithms are not open access which hampers the implementation and prevents incorporation of new algorithms developed by researchers. This disadvantage can be overcome by the use of distributed processing algorithms, such as the Matlab code (The MathWorks, Natick, MA, USA) provided in (Eilers, 2004).

The experimental data indicate a variation in sample size that was injected onto the GC column. This variation was derived from the differences in intensity of the peak corresponding to the naphthalene standard (not shown). This emphasizes the necessity for normalisation of data. MetAlign allowed the normalisation to one specific mass fragment. But, this procedure does not allow the selection of more fragments which was desirable in our case since similar fragments were located at similar retention times (not shown). In addition, it can be seen from the chromatographic profiles that there is need for baseline correction (Fig. 7.1 and Fig. 7.4A). The baseline correction algorithm performed by MetAlign turned out to produce an acceptable result (Fig. 7.4B). This seems important as baseline correction is imperative for the automatic pre-processing of chromatographic data (Christensen *et al.*, 2005).

The observed variation in scan numbers of signals points to the presence of unwanted peak drifts betweens samples (Fig. 7.3 and Fig. 7.4). From literature it is known that small peak drifts are common in chromatographic data. These drifts are known to all chromatographers and are due to changes in the columns during use, minor changes in mobile phase composition, drift in the instrument or interaction between analytes (Nielsen *et al.*, 1998). Small retention time drifts in the order of 1 – 250 scans were observed in our data. The data should therefore be corrected for this drift to improve the measurement of marker compounds. Several algorithms are described in literature for the alignment of chromatographic data. The alignment procedure, also referred as peak matching, can be done with COWtool software (Nielsen *et al.*, 1998). This method relies on piecewise linear correlation optimised warping (COW). A second commonly used alignment algorithm is based on dynamic time warping (DTW). Tomasi *et al.* (2004) studied these two different algorithms –COW and DTW- as pre-processing steps for chromatographic data. They concluded that time alignment corrections should be handled with great care and pointed to difficulties with respect to the judgement of performance. We also experienced difficulties to assess the result of an alignment as produced by MetAlign. It seems a generally accepted benchmark method is lacking. Lin *et al.* (2005) determined whether the inconsistence was due to amplitude differences or phase variations using a “lobster plot”. This graphical evaluation of the result of aligning could also be applied to our data. However, it should be kept in mind that this procedure becomes time consuming and more subjective when sample sizes increase.

Proper data processing software extents the use of GC-MS instruments to other agricultural application such as quality control. Potato-tubers are among the agricultural products that could be checked for quality-loss based on the analysis of emitted VOCs (Varns and Glynn, 1979, Waterer and Pritchard, 1984). Recently, this method was successfully applied at laboratory scale to monitor quality aspects of several other agricultural products including milk, meat, vegetables, grains, and fruits (Hettinga *et al.*, 2008, Moalemiyan *et al.*, 2006, Vikram *et al.*, 2006, Vikram *et al.*, 2004). Appropriate processing software is required but GC-MS instruments also need to become less expensive before they can be applied in an agricultural setting.

CONCLUSION

This research is a response to the need of automatic data processing for GC-MS based health monitoring. The frequent presence of low intensity signals, the peak shift and the occurrence of baseline drift in our data emphasize this need of processing the data. The processing of data improved the automated interpretation of the chromatographic data and allowed us to determine the concentrations of health associated VOCs in a greenhouse.

CHAPTER 8

Synthesis, conclusion, and outlook

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SYNTHESIS

This thesis reports on the outcome of a study on the detection of pathogen infection through volatile organic compounds (VOCs) emitted from plants. The main research objective of this study was to investigate whether plant-emitted VOCs can be used to detect a pathogen infection in a large-scale greenhouse. The pathogenic fungus *Botrytis cinerea* and the plant species tomato (*Lycopersicon esculentum*) were selected as model organisms. Based on this choice, three main research questions were formulated: (1) What is the effect of a *B. cinerea* infection on the emission of VOCs from tomato? (2) Are *B. cinerea* induced emissions of tomato specific for the infection with this pathogen? (3) Are *B. cinerea* induced concentrations of VOCs detectable in large-scale greenhouses?

Effect of a *Botrytis cinerea* infection on the emission of VOCs from tomato

Severe *B. cinerea* infections resulted in a large increase in the emission of alcohols and aldehydes a few hours after inoculation and mild infections resulted in a small increase in the emissions of alcohols and aldehydes several hours after inoculation (CHAPTER 3). Once the emission of these type of VOCs reached their maxima, they reduced to values below detection limits within a few hours.

The alcohols and aldehydes were undetected in experiments on *B. cinerea* infected leaves (CHAPTER 2). Why were these compounds undetected in experiments on detached leaves? A first explanation could be that the 22 h delay between inoculation and collection of samples prevented the detection of a burst of alcohols and aldehydes before that period of time. A second explanation arises from the physicochemical properties of VOCs. Alcohols and aldehydes are water soluble; maybe they were dissolved in the water which was added into the Petri dishes to prevent dehydration of the leaves. A third option to consider is the sorbent used during sampling since the choice of sorbent is crucial for ensuring efficient concentration of volatiles (Agelopoulos and Pickett, 1998). In experiments on detached leaves, part of the samples were obtained using Tenax, a sorbent commonly used to concentrate the alcohols and aldehydes of interest (Agelopoulos *et al.*, 1999). However, most of the samples were obtained by the use of poly-dimethylsiloxane (PDMS) as solid sorbent. This type of sorbent is suitable for concentrating non-polar semi-volatile compounds, but not for the polar alcohols and aldehydes (Deng *et al.*, 2005).

Besides emissions of alcohols and aldehydes, severe *B. cinerea* infections resulted in the increased emission of mono- and sesquiterpenes from whole tomato plants (CHAPTER 3). This was not the case when whole plants showed mild symptoms of an infections by *B. cinerea*. This was also not the case for detached leaves upon infection (CHAPTER 2). Why remained the emission of mono- and most sesquiterpenes stable upon infection of detached leaves? Probably, the severity of infection was insufficient to alter the emission of these terpenes considerably; an opinion supported by the work of Mithöfer *et al.* (2005) who showed that the extent of damage has an effect on the emission of the monoterpenes ocimene and linalool from detached lima bean (*Phaseolus lunatus*) leaves.

The sesquiterpene *α*-copaene was the one exception which proved to be predominantly emitted from infected tomato leaves (CHAPTER 2). In contrast to the increased emission of the sesquiterpene *α*-copaene from infected leaves, the results showed mixed findings in the case of infected whole plants. Whole plants, non infected as well as infected did not show a consistent change in emission of *α*-copaene. In some cases it increased, sometimes it decreased or remained constant at same temperature and light regime. This difference might have been caused by the use of whole plants. Such differences between detached leaves and whole plants were already described by Schmelz *et al.* (2001) who demonstrated that the results of assays using excised tissues should be cautiously interpreted when considering whole-plant models.

Besides alcohols, aldehydes, monoterpenes, and sesquiterpenes, *B. cinerea* infections affected the emission of the ester-substituted phenol methyl salicylate (CHAPTER 3). Non infected plants showed a small but increasing emission of methyl salicylate during the three days period, probably as a result of stress due to enclosure of plants. Infected plants showed a larger increase in the emission of methyl salicylate compared to non infected plants (Jansen *et al.*, 2009d). No obvious correlation between the severity of infection and the increase in methyl salicylate could be established. Methyl salicylate was undetected or present in trace level amounts in the experiments on detached leaves (CHAPTER 2). This compound can be efficiently trapped on PDMS (Deng *et al.*, 2004a) and the sampling procedure itself could therefore not be the reason for the absence or low presence. Maybe, the compound was dissolved into water or differences between detached leaves and whole plants caused this difference.

Finally, emissions of the homoterpene (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were affected by a *B. cinerea* infection (CHAPTER 3). Non infected plants showed a small but increasing emission of TMTT during the three days period, probably as a result of stress due to enclosure of plants. Infected plants showed a larger increase in the emission of TMTT compared to non infected plants. Similar to methyl salicylate, no obvious correlation between the severity of infection and the increase in TMTT emissions could be established. TMTT was undetected or present in low amounts in the experiments on detached leaves (CHAPTER 2). It is unclear whether this compound can be efficiently concentrated on PDMS but likely the compound TMTT was concentrated and then incorrectly identified as squalene (Deng *et al.*, 2004a) and the sampling procedure itself could therefore not be the reason for the absence or low amounts.

In short, the emission of several VOCs increased upon infection of whole plants which did not increase upon infection of detached leaves and *vice versa*. But, given the thorough study on whole plants, the presence of alcohols and aldehydes was regarded as reliable indicators of a *B. cinerea* infection. On these grounds, increasing mono- and sesquiterpene emissions, apart from α-copaene, and increasing methyl salicylate and TMTT emissions were also regarded as reliable indicators of a *B. cinerea* infection. Furthermore, compared to research on detached leaves, the study on whole plants resembled conditions more comparable to greenhouse horticulture.

As mentioned before, the results obtained during the laboratory tests on whole plants were regarded as reliable and therefore served as basis for follow up studies. No additional time was spent on the inspection of data obtained from greenhouse air after infection of plants with *B. cinerea* in order to search for novel compounds. But, it can be questioned whether this was a good strategy since plants in a greenhouse may emit additional compounds due to *e.g.* differences in light intensity and/or differences in plant age.

In the laboratory tests on whole plants, plants were infected by spraying them with a solution containing *B. cinerea* spores. However, in practice, infections in tomato are usually restricted to stem wounds (Shtienberg *et al.*, 1998) which generally originate from pruning and removal of side shoots. To mimic these conditions, it might have been better to use the method as

described by Finkers*, et al.* (2007). They infected tomato plants by making incisions into the stem. Then, they inoculated the wounds with agar plugs containing a culture of *B. cinerea* after which the wounds were covered with tape to ensure high humidity. Besides a more realistic way of inoculation, this procedure has the advantage that it eliminates the need of a high air humidity and therefore prevents the trapping of moist air which often results in technical problems in VOC analysis.

Specificity of *Botrytis cinerea*-induced emissions of VOCs from tomato

In the previous section, the effect of a *B. cinerea* infection on the emission of VOCs from tomato was discussed. However, throughout the study, it became obvious that other factors occur in tomato production greenhouses that also affect the emission of VOCs from tomato. These factors should be considered since a *B. cinerea* specific detection is only feasible if such an infection consistently results in the emission of unique VOCs. Table 8.1 provides an overview of VOCs and literature in which emissions of these VOCs have been reported to increase upon biotic and/or abiotic stresses of intact tomato plants or detached tomato leaves. None of the VOCs listed in Table 8.1 can be exclusively linked to one particular stress of tomato. Therefore, it is improbable that the causal agent of plant stress can be identified as a *B. cinerea* infection based on these VOCs only.

Table 8.1 Volatile organic compounds (VOCs) emitted from whole, intact tomato plants or detached leaves and the biotic and/or abiotic stresses responsible for the increase in VOC emissions. Numbers in superscript specify the references used.

VOCs	**Biotic and abiotic stresses that increase VOC emissions from tomato**
ALCOHOLS	
(*Z*)-3-hexenol	*Botrytis cinerea*[1], *Spodoptera littoralis*[2], *Liriomyza huidobrensis*[5], *Spodoptera exigua*[8], *Manduca sexta*[12], *Macrosiphum euphorbiae*[9], ozone[13], *Helicoverpa armigera*[14]
ALDEHYDES	
(*E*)-2-hexanal	*Botrytis cinerea*[1], *Spodoptera littoralis*[2], *Liriomyza huidobrensis*[5], *Spodoptera exigua*[8], *Manduca sexta*[12], ozone[13], *Helicoverpa armigera*[14]

MONOTERPENES	
linalool	*Spodoptera littoralis*[2,7], *Tetranychus urticae*[3]
α-pinene	*Botrytis cinerea*[1], *Spodoptera littoralis*[2,7], *Spodoptera exigua*[8], *Macrosiphum euphorbiae* [9], *Oidium neolycopersici*[11], *Manduca sexta*[11,12], ozone[13]
α-terpinene	*Botrytis cinerea*[1], *Spodoptera littoralis*[2,7], *Spodoptera exigua*[8] *Oidium neolycopersici*[11], *Manduca sexta*[11,12], ozone[13]
SESQUITERPENES	
β-caryophyllene	*Spodoptera littoralis*[2,7], *Spodoptera exigua*[8], *Macrosiphum euphorbiae* [9], *Manduca sexta*[12], ozone[13]
α-copaene	*Botrytis cinerea*[10], *Spodoptera littoralis*[2]
HOMOTERPENES	
(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	*Botrytis cinerea*[1], *Tetranychus urticae*[3,6], *Liriomyza huidobrensis*[5], *Manduca sexta*[12], ozone[13]
PHENOLICS	
methyl salicylate	*Botrytis cinerea*[1], *Spodoptera littoralis*[2], tobacco mosaic virus[4], *Tetranychus urticae*[3,6], *Manduca sexta*[8,12], *Macrosiphum euphorbiae* [9], ozone[13]

[1] Jansen *et al.* (2009c); [2] Vercammen *et al.* (2001); [3] Ament *et al.* (2004); [4] Deng *et al.* (2004b); [5] Wei *et al.* (2007); [6] Dicke *et al.* (1998); [7] Maes *et al.* (2003), [8] Thaler *et al.* (2002); [9] Sasso *et al.* (2007); [10] Thelen *et al.* (2006); [11] Laothawornkitkul *et al.* (2008); [12] Farag and Paré,(2002); [13] Miebach (personal communication); [14] Deng *et al.* (2005).

The overview in Table 8.1 shows that the most common stress-induced VOCs are unspecific. On the other hand, literature offers some evidence for specific signals in the volatile blend of stressed plant species. For instance, the ability of host-seeking insects to recognize and respond to certain VOCs and to differentiate them from background VOCs indicates that insect-damaged plants emit VOCs that are distinguishable from those emitted in response to other types of damage or those emitted from undamaged plants (Paré and Tumlinson, 1999). Probably, such specific signals are based on the temporal and spatial variations of VOCs at concentration levels far below the detection limits of commonly used analytical instruments including the instruments used in our experiments.

The overview in Table 8.1 is perhaps not complete, but the fact that emissions of many of the same substances increase upon different stressors suggests a general plant response by similar underlying mechanisms. Some of these mechanisms are briefly discussed below.

B. cinerea infected plants emitted several alcohols and aldehydes. These VOCs are denoted as lipoxygenase products. They originate from the oxidative cleavage of C_{18}-fatty acids in the presence of oxygen and enzymes such as lipoxygenases. They are emitted upon damage of cell membranes (that contain fatty acids) and are known to us as the characteristic smell that appears after cutting grass. Many biotic stresses such as herbivore feeding may result in damage of cell membranes which clarifies the numerous herbivore induced increases in emissions of the lipoxygenase products (Table 8.1).

An additional source for lipoxygenase products that should be considered is plant debris such as excised shoots after pruning. Such debris is nearly always present in a greenhouse and may then increase the concentrations of lipoxygenase products due to drying. Also nearby field crops are expected to be sources of lipoxygenase products, especially upon harvest or stress. These events are extremely difficult to predict. Consequently, the emission of lipoxygenase products is unsuitable for a specific detection of *B. cinerea* infections in tomato production greenhouses and probably also not suitable for the detection of a general stress response of the crop.

Severely *B. cinerea* infected tomato plants emitted larger quantities of mono- and sesquiterpenes compared to non infected plants. Also spraying with an aqueous solution containing *B. cinerea* spores resulted in an increase of mono- and sesquiterpene emissions (Jansen, 2006). The emissions peaked within 1 h after spraying and returned to initial levels within 2 to 3 h. This burst was attributed to the damage of glandular trichomes as a result of spraying by which water droplets smack onto the stems and leaves. These trichomes are outgrowths of the plant epidermis and collectively constitute the pubescence of the plant surface. To study the importance of trichomes in more detail, we stroked the full length of the stem of one of the plants enclosed in the chamber. Also this treatment resulted in a large burst of mono- and most sesquiterpene emissions. These results were not provided in CHAPTER 3. However, this result confirmed the importance of trichomes as source of plant volatiles and

supports the work of van Schie *et al.* (2007) who demonstrated that trichomes of tomato store in their interior considerable amounts of mono- and sesquiterpenes.

A burst in mono- and sesquiterpene emissions was also observed at greenhouse-scale when fruits were harvested (CHAPTER 4), and side shoots were removed (CHAPTER 4). Almost certainly, every other crop operation will affect the emission of mono- and sesquiterpenes. Also temperature determines the emission of mono- and sesquiterpenes. Harvesting fruits, removal of side shoots, other crop operations, and fluctuations in temperature occur often in greenhouse practice. Moreover, many biotic stresses such as herbivore feeding may result in damage of trichomes which clarifies the numerous herbivore induced increases in emissions of mono- and sesquiterpenes (Table 8.1). Consequently, an increase in the emission of mono- and sesquiterpenes is unsuitable for a specific detection of *B. cinerea* infections in tomato production greenhouses and probably also not suitable for the detection of a general stress response of the crop.

B. cinerea infected tomato plants emitted larger quantities of methyl salicylate. Such an increase was also reported upon stress of tomato as a result of at least seven different biotic and abiotic stressors (Table 8.1). Therefore, increased emissions of methyl salicylate are not specific for a *B. cinerea* infection. The slight increase in the emission of methyl salicylate from enclosed control tomato plants as reported in CHAPTER 3 was probably a result of stress due to enclosure. This effect was also mentioned by Ament (2006), providing an additional hint that increased emissions of methyl salicylate are not specific to any type of stress but rather a general stress response.

The concentrations of methyl salicylate remained stable after stroking of stems, after removal of side shoots, and after picking fruits (CHAPTER 4). Three reasons may account for this. First, *B. cinerea*-derived elicitors were absent in the above mentioned treatments. These elicitors may play an important role in the induction of methyl salicylate emissions from tomato plants. Second, the damages incurred to the plants were a momentary type of damage while *B. cinerea* probably results in a continuous type of damage. There is an increasing body of evidence suggesting that the time course of damage has an effect on the emissions of VOCs from plants. Third, VOCs were measured directly after the damage stopped. There are several

examples in literature in which the emissions of volatiles, such as methyl salicylate, increase, but with a time delay of several hours after the stress application.

The fact that stroking, removal of side shoots, and picking fruits did not affect the concentration of methyl salicylate is beneficial since then, methyl salicylate allows the discrimination between plant stress and crop operations. Ultimately, an increase in the concentration of methyl salicylate might serve as an effective warning sign for the presence of *B. cinerea* since the diversity of stress factors that occur in a tomato production greenhouse is often limited. However, it should be noted that methyl salicylate emission from tomato is also light dependent (Farag and Paré, 2002, Maes and Debergh, 2003). As light will fluctuate in a greenhouse, this will have to be taken into account when correlating increased methyl salicylate concentrations in the greenhouse atmosphere to any type of plant stress.

The increased emission of the homoterpene TMTT after inoculation remains poorly understood due to the limited amount of information available, and uncertainties about this information. For instance, few researchers indicate increased TMTT emissions from tomato upon stress, whereas others did not (Table 8.1). The interpretation of TMTT emissions is also complicated by the fact that several researchers observed large amounts of TMTT in the headspace of control tomato plants while other researchers did not observe this compound at all, or failed to identify it correctly.

Detectability of *Botrytis cinerea*-induced concentrations of VOCs in large-scale greenhouses

To find out whether *B. cinerea* is detectable in large-scale greenhouses, it is important to know the induced increase in concentrations as well as the precision and detection limits of analytical instruments. Therefore, we developed a model to predict whether volatiles can be used to detect a *B. cinerea* infection in a large-scale tomato production greenhouse with a volume of 5×10^4 m^3 containing 2.2×10^4 plants (CHAPTER 6). The precision and detection limits of a gas chromatograph (GC) coupled to a mass spectrometer (MS) or flame ionization detector (FID) were compared with the *B. cinerea*-induced increase in concentration of the lipoxygenase product (*Z*)-3-hexenol, the trichome damage related VOCs *α*-pinene, *α*-terpinene and *β*-caryophyllene, and the volatile plant hormone methyl salicylate to

determine the appropriateness of these instruments for measuring the increase. The model was used to predict the effect of the fraction of infected plants and the effect of the air exchange rate on the *B. cinerea*-induced increase in concentration.

Independent of the air exchange rate, the *B. cinerea*-induced increases in concentration of (*Z*)-3-hexenol is detectable in a large-scale tomato production greenhouse when 0.5% or more of the plants are infected. Independent of the air exchange rate and independent of the fraction of infected plants, the *B. cinerea*-induced increases in concentration of *α*-pinene, *α*-terpinene and *β*-caryophyllene are undetectable in a large-scale tomato production greenhouse. The *B. cinerea*-induced increase in concentration of methyl salicylate is detectable in a large-scale tomato production greenhouse when at least three conditions are met: (a) windows are fully opened, and (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 1 h, and (c) 5% of the plants are infected. The *B. cinerea*-induced increase in concentration of methyl salicylate is also detectable when (a) windows are closed, and (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 6 h, and (c) 5% of the plants are infected.

These findings are bases on the momentaneous concentrations of VOCs. In practice, VOCs are often pre-concentrated to achieve the detection limits of commonly applied analytical instruments. The period of time required for pre-concentration depends on the concentration of the VOCs of interest in the air. Also the separation of VOCs in the mixture requires a certain amount of time. A sensitivity analysis should include the separation and pre-concentration periods to determine the detectability of *B. cinerea*-induced increase in concentrations of VOCs in large-scale greenhouses. Such analysis should also include the period of time in which the *B. cinerea*-induced increase in emission of VOCs from a certain proportion of plants is above the baseline level emission of healthy plants. Furthermore, a sensitivity analysis should include the emission flux densities of methyl salicylate by healthy tomato plants. These values were obtained under laboratory conditions. It is doubtful whether laboratory conditions are suitable to determine methyl salicylate emissions from healthy plants since stress due to enclosure of tomato plants -a prerequisite for analysing plant emissions at the laboratory scale- also led to increased emissions of methyl salicylate (Ament, 2006).

Analytical instruments based on GC-MS and GC-FID with a precision of 10% relative standard deviation and a detection limit of 1 nmol m^{-3} were used as a reference. These type of instruments are routinely used to detect air contaminants in field settings (Greenberg *et al.*, 1994, Smith *et al.*, 2005) and to monitor biogas tower reactors for the presence of potentially toxic VOCs (Matz *et al.*, 1998). Besides GC-MS and GC-FID, electronic noses (e-noses) are also widely used to detect plant-emitted VOCs in air (Kunert *et al.*, 2002). In general, they are not useful for the identification and quantification of individual components (Gardner and Bartlett, 1999). However, the identification of the volatiles being emitted may not be needed if the comparison and recognition of patterns in the volatile profile are sufficient for crop health monitoring through the analysis of plant-emitted volatiles. Such a profile can be obtained through the use of sensor arrays. This converges with research on volatile based inspection of trees based on e-nose systems which rely on the recognition of fingerprints of volatiles released from them. For instance, a prototype device incorporating three metal oxide sensors was able to detect basal stem rot disease of oil palm (*Elaeis guineensis* Jacq.) infected by the fungus *Ganoderma boninense* (Markom *et al.*, 2009).

A combination of the marker-compound-approach with the e-nose technique can result in e-nose systems that have the ability to quantify VOC concentration in air as demonstrated for the differentiation of fresh and rancid butter based on volatiles (Hofmann *et al.*, 1997). This development seems to be quite promising. The remaining drawback of e-noses based on sensor arrays is that the threshold of determination of most of these systems is in the low ppmv-range. However, this drawback can be overcome by utilization of pre-concentration techniques. Such a combination of a gas-chromatographic system equipped with a pre-concentration unit and e-nose was successfully applied to detect plant emitted volatiles in a small cuvette (Kunert *et al.*, 2002). However, the reported limits of detection for this instrument (see Watkins and Wijesundera, 2006), are several orders of magnitude less than required for the in CHAPTER 6 predicted concentrations of *B. cinerea*-induced volatiles in a large-scale greenhouse.

More recently, biosensors have been developed to identify and quantify low levels of VOCs in ambient air. A biosensor is a particular type of chemical sensor that uses the recognition properties of biological components in the sensitive layer. Today even whole animals or certain organs of animals are used in biosensors. Especially insects are seen as suitable model

to develop biosensors for gas analysis. Several studies attempted to quantify the sensitivity of insects to certain volatiles. For instance, trained wasps responded to 3-octanone, myrcene, cadaverine and putrescine at concentrations in the order of 10 ppbv (Rains *et al.*, 2004), and a biosensor based on insect antennae responded to (*Z*)-3-hexenol at a concentration of 10 ppbv (Schütz *et al.*, 1996). These sensitivities are several orders of magnitude less than required. Besides the demand for an increase in sensitivity, there are numerous other methodological and biological hurdles that needs exploration before sensors based on insects can be used in horticultural practice.

CONCLUSIONS

The research presented in this thesis has led to the following conclusions.

(1) Tomato plants emit different types and amounts of volatiles during infection by *B. cinerea*. The main effects are the burst of lipoxygenase products and the increase in emissions of monoterpenes, sesquiterpenes, methyl salicylate and TMTT. The burst of lipoxygenase products is probably the result of damage to cell membranes. The increase in emissions of monoterpenes and sesquiterpenes is probably the result of damage to glandular trichomes. The increase in emission of methyl salicylate and TMTT is not directly related to cell membrane or trichome damage but probably the result of a systemic plant response as a result of stress.

(2) Based on model predictions, the *B. cinerea*-induced increase in concentration of lipoxygenase products is detectable in a large-scale greenhouse when 0.5% of the plants are infected. However, many additional sources of lipoxygenase products exist including plant debris and nearby field crops especially upon harvest and stress. Plant debris is nearly always present and harvest and/or stress of nearby crops is extremely difficult to predict. Consequently, lipoxygenase products can probably not be used to detect a *B. cinerea* infection in a large-scale greenhouses.

(3) Based on model predictions, the *B. cinerea*-induced increase in concentration of mono- and sesquiterpenes cannot be detected in a large-scale tomato production greenhouse. Furthermore, crop operations will almost certainly affect the concentration of mono- and sesquiterpenes. These crop operation occur often. Consequently, mono- and

sesquiterpenes can probably not be used to detect a *B. cinerea* infection in a large-scale greenhouses.

(4) Based on model predictions, the *B. cinerea*-induced increase in concentration of methyl salicylate is detectable in a large-scale tomato production greenhouse when at least three conditions are met: (a) windows are opened, and (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 1 h, and (c) 5% of the plants are infected. The *B. cinerea*-induced increase in concentration of methyl salicylate is also detectable when (a) windows are closed, and (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 6 h, and (c) 5% of the plants are infected. Consequently, methyl salicylate can probably be used to detect a *B. cinerea* infection in a large-scale greenhouses. However, the *B. cinerea*-induced increase in concentration of methyl salicylate is not specific for a *B. cinerea* infection of tomato. Therefore, it will be impossible to identify the stressor as *B. cinerea* based on methyl salicylate emissions only.

OUTLOOK

In this research, much insight has been gained into volatile based crop monitoring. However, it is clear that this topic is in its infancy and far from being completely understood. Therefore research effort in the following areas is suggested.

When we received the referee rapports of (Jansen *et al.*, 2009b) an anonymous referee wrote: 'If the goal is to detect an early fungal infestation wouldn't specific VOCs emitted by the fungus itself be a better way to diagnose the infection? It is known that fungi can emit a wide range of unique VOC signatures that could aid in detecting an early infestation'. This question is an interesting one and addresses an important point for future research.

Future research may involve low-molecular VOCs to monitor crop health at greenhouse scale. In this study, we focussed on the mid-molecular weights VOCs in the range of C_5-C_{24}. However, an infection of tomato plants with *B. cinerea* probably also affects the emission of low-molecular weight VOCs (< C_5). For instance, emissions of nitric oxide (NO), hydrogen peroxide (H_2O_2), ethylene (C_2H_4), ethane (C_2H_6), acetaldehyde (C_2H_4O), and ethanol (C_2H_5OH) from diverse plant species were found to increase upon stress exposure. Depending

on the source- and sink strengths of these VOCs, they may be used as indicator of plants stress at greenhouse scale, but probably not to identify the stressor itself.

Due to the high costs, we are years away from having sensitive and precise analytical instruments in horticultural practice. But, the ongoing expansion and intensification of greenhouse production, and the concern among consumers about the potential intake of pesticide residues on fruits and vegetables will support the prospected application of plant health monitoring in a commercial setting. Another point for future research is therefore the development of sensitive, precise, but also affordable instruments, specifically designed for application in horticulture practice. Four steps should then be considered. First, the collection and pre-concentration of the plant emitted VOCs. Second, the separation of the plant emitted VOCs in the mixture. Third, the identification, and/or quantification of the separate VOCs. Fourth, the automatic processing of data. Colorimetric tubes based on a chemical reaction generating a colour change may offer an alternative cost-effective approach to measure the concentration of important stress associated VOCs such as methyl salicylate.

To determine the potential of volatile based crop monitoring, it is necessary to perform semi- and large-scale experiments. Care should then be taken because such experiments will be influenced by the inherent variability present in crops grown in practice. Especially the importance of VOC transfer into water should be studied, more specifically the role of condensation on dehumidifiers versus the role of condensation on the glass cover.

Finally, the effect of other stressors on the emission of VOCs from tomato and other plant species that are common in greenhouse horticulture should be studied. Especially infections by root pathogens seems an important plant health problem to study because the effect of these types of infection are difficult to see by the naked eye.

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SUMMARY

Crops in greenhouses are typically cultivated as a monoculture which means that one crop species is grown. This crop is usually grown at a high plant density, and because the climate can be controlled, it is possible to cultivate crops on a year-round basis. Year-round monoculture crops at high plant densities are susceptible to diseases due to the ease with which most diseases can spread. Crop protection chemicals are routinely used to reduce the incidence and spreading of diseases. But, the consumers are demanding a reduction in the use of crop protection chemicals in their foods. In addition, the exposure of crop protection chemicals has a negative effect on the health of greenhouse workers and also pollutes the environment.

Local treatments of plants with reduced amounts of crop protection chemicals are sufficient if disease symptoms are discovered at an early stage. Crops are therefore regularly inspected for these symptoms. These inspections are important, especially in large-scale greenhouses because diseases can then spread quickly over large areas. But, inspections are also expensive because they require considerable amounts of time and skilled personal. As a result, there is a demand for a system to inspect crops which discovers diseased plants, preferably at an early stage. One proposed concept is the measurement of volatile organic compounds (VOCs) emitted from plants. This concept is based upon the numerous laboratory studies which revealed that pathogen infections have an effect on the volatile blend released by plants. This provides the opportunity to use plant-emitted VOCs for detection of plant disease.

The main research objective of this study was to investigate whether plant emitted VOCs can be used to detect a pathogen infection in a large-scale greenhouse. The study was focused on the detection of *Botrytis cinerea* infections in tomato (*Lycopersicon esculentum*). *B. cinerea* was selected as a model pathogen because it is a well-known cause for considerable damage in a broad range of plant species including tomato and strawberry. Tomato was selected because greenhouse production of tomato is an economically important industry worldwide.

Knowledge about the emissions of VOCs from *B. cinerea* infected and healthy tomato plants is required to assess whether a *B. cinerea* infection is detectable through plant-emitted VOCs. A *B. cinerea* specific detection is feasible if an infection consistently results in the emissions of unique VOCs. To determine their uniqueness, also other factors which might have an effect

on VOC emission from tomato were studied including crop operations, other stressors, but also environmental factors such as an increase in temperature or light intensity. The effect of these factors on VOC emission determine the reliability of VOCs as source of information to detect a *B. cinerea* infection. An important aspect are the concentrations of VOCs in a large-scale greenhouse during a *B. cinerea* infection. They are essential parameters to decide on when evaluating analytical instruments for sensing VOCs in large-scale greenhouses. The specific research questions addressed in this thesis were:

1. What is the effect of a *B. cinerea* infection on the emission of VOCs from tomato?
2. Are *B. cinerea* induced emissions of VOCs from tomato specific for the infection with this pathogen?
3. Are *B. cinerea* induced concentrations of VOCs detectable in a large-scale greenhouse?

The initial phase of the research was used to investigate the emissions of VOCs from detached tomato leaves which were individually enclosed in Petri dishes (CHAPTER 2). The volatiles were both statically and dynamically concentrated during the sampling of the air surrounding the non-infected and *B. cinerea* infected leaves. The analysis of samples was conducted by gas chromatography (GC), mass spectrometry (MS) and flame ionisation detection (FID). Results from this phase gave a first impression of VOCs emitted from healthy and *B. cinerea* infected tomato plants. During this phase, hands-on experience was gathered with instruments for sampling and analysis. Results from this phase showed that detached leaves emit higher amounts of the sesquiterpene α-copaene upon infection with *B. cinerea*.

In follow up experiments, whole plants were used to investigate their emissions (CHAPTER 3). Per experiment, several non infected or *B. cinerea* infected plants were enclosed for three days in a chamber with temperature, humidity, light intensity, and CO_2 control. Clean air was drawn into this chamber and every hour, the outgoing air was dynamically sampled after which the sample was directly analysed using GC-MS. During this period, at least 40 VOCs were identified that were emitted from tomato plants. Measurements of leaf area and calibrations by means of reference compounds were used to normalize the emissions of the fifteen most dominant compounds into emissions per unit leaf surface and time. These flux

densities showed large variations between replicate studies in case of non infected as well as *B. cinerea* infected plants.

In contrast to the experiments on detached *B. cinerea* infected leaves, alcohols and aldehydes were detected in experiments on *B. cinerea* infected plants. These compounds are denoted as lipoxygenase products; they originate from the oxidative cleavage of C_{18}-fatty acids in the presence of oxygen and enzymes such as lipoxygenases. These products are emitted from damaged plants in which damage of cell membranes play an important role. Most dominant was the lipoxygenase product (*Z*)-3-hexenol which showed flux densities up to 0.5 nmol m^{-2} s^{-1}. The emission of lipoxygenase products was explained by the *B. cinerea* induced damage to cell membranes. Emissions of these compounds were larger and started earlier in case of severe infections compared to mild infections. The increase in emissions of lipoxygenase products was observed several hours after inoculation which demonstrates that these compounds provide an early indication of cell membrane damage, even before symptoms of a *B. cinerea* infection became visible.

Non infected as well as *B. cinerea* infected tomato plants emitted a number of monoterpenes and sesquiterpenes including *α*-copaene. But, in contrast to infected detached leaves, the emission of *α*-copaene did not dominate in case of infected intact plants. This difference was explained by the use of intact plants instead of detached leaves. The diurnal rhythm in the flux densities of monoterpenes and sesquiterpenes were mainly ascribed to differences in leaf temperature. Increased flux densities of monoterpenes were observed from severely infected plants with flux densities up to 1 nmol m^{-2} s^{-1}. The ratio in flux densities between the monoterpenes remained constant which suggests a similar generic mechanism, probably damage to glandular trichomes.

Non infected and *B. cinerea* infected plants emitted methyl salicylate and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraen (TMTT). The diurnal rhythm in the flux densities of methyl salicylate and TMTT were mainly attributed to differences in light intensity. Non infected plants showed small but increasing flux densities of methyl salicylate and TMTT during the three days period, probably as a result of stress due to enclosure of plants. Infected plants showed a larger increase in the flux densities of methyl salicylate and TMTT compared to non infected plants in the order of nmol m^{-2} s^{-1}. No obvious correlation between the severity of

infection and the increase in methyl salicylate and TMTT emissions could be established. In the literature, these compounds are defined as phytohormones and emission increases upon biotic and abiotic stresses are well known for several plant species.

In CHAPTER 4, experiments are described which were conducted in a small experimental greenhouse containing 60 plants situated on a floor area covering 42 m^2. These plants were artificially damaged throughout a period of two months to simulate a *B. cinerea* infection. The damage consisted of stroking the stems to damage glandular trichomes and removal of side shoots to damage cell membranes. In addition, we studied the effect of fruit picking on the concentrations of plant-emitted VOCs in greenhouse atmosphere. Air samples were collected at three locations within the greenhouse and subsequently transferred to the laboratory for GC-MS analysis. In all cases, the concentrations of volatiles were nearly similar in between the three locations which was explained by the high internal air circulation of 2×10^4 m^3 of air per hour.

At least nine monoterpenes, four sesquiterpenes, three phenolics, and one homoterpene were detected in air samples obtained before damage of the plants. These compounds were known to be emitted from tomato and detected in our previous laboratory experiments. The monoterpene *β*-phellandrene was always present at the highest concentration. When plants were seven weeks old, the concentration of this VOC was approximately 0.06 nmol per mol of air before treatment. This concentration was raised to approximately 0.14 nmol per mol of air when plants were twelve weeks old. Stroking of the stems, removing the side shoots and fruit picking resulted in an increase in the concentrations of all mono- and most sesquiterpenes up to 60-fold which was expected since these volatiles are well known constituents of glandular trichomes. The ratio between the concentrations of the monoterpenes *β*-phellandrene and 2-carene remained constant after stroking of the stems while the ratio between de concentrations of the monoterpene *β*-phellandrene and the sesquiterpenen *β*-caryophyllene changed. This offers the opportunity to detect trichome damage on the basis of ratio's between individual volatiles.

The concentrations of the *B. cinerea*-related volatiles *α*-copaene, methyl salicylate, and TMTT remained stable after stroking of stems, after removal of side shoots, and after picking fruits. Three reasons may account for this. First, *B. cinerea*-derived elicitors were absent in

the above mentioned treatment. These elicitors may play an important role in the induction of *α*-copaene, methyl salicylate and/or TMTT emissions from tomato plants. Second, the damages incurred to the plants were a momentary type of damage while *B. cinerea* probably results in a continuous type of damage. There is an increasing body of evidence suggesting that the time course of damage has an effect on the emissions of VOCs from plants. Third, VOCs were measured directly after the damage stopped. There are several examples in literature in which the emissions of volatiles, such as methyl salicylate, increase, but with a time delay after the stress application.

In contrast to stroking and fruit picking, shoot removal resulted in the detection of the lipoxygenase product (*Z*)-3-hexenol in greenhouse atmosphere at concentrations between 8 and 20 pmol per mol of air. This VOC is expressing cell membrane damage and this type of damage is therefore detectable in a small-scale greenhouse.

The experiment described in CHAPTER 5 was conducted in the same greenhouse as the greenhouse described in CHAPTER 4. In this experiment, all 60 plants were inoculated with *B. cinerea*. The objective of this experiment was to determine the effect of inoculation on the concentrations of the typical cell membrane damage-induced lipoxygenase products, on the concentration of the typical trichome damage-induced monoterpenes and sesquiterpenes, and on the concentration of the volatile phytohormones methyl salicylate and TMTT. Upon inoculation, the greenhouse air was sampled semi-continuously with a one hour time interval until 72 hours after inoculation. The samples were transferred to the laboratory and analysed using GC-MS. Ten leaves were randomly selected to monitor the visible symptoms of infection. The severity of these visual symptoms was assessed at 0, 24, 48, and 72 hours after inoculation.

Lipoxygenase products were undetected after inoculation of the plants. The absence of lipoxygenase products was explained by the mild *B. cinerea* symptoms and/or the transfer of these products into free water. The concentration of all monoterpenes, most sesquiterpenes, and TMTT remained stable after inoculation while the concentration of *α*-copaene fluctuated according to the day/night rhythm. This fluctuation probably resulted from the light dependant synthesis of *α*-copaene which demonstrates that plant volatiles can be used to measure the biosynthesis of compounds inside a greenhouse grown crop in a non-invasive way. The

concentration of methyl salicylate directly after inoculation was approximately twenty picomol per mol air. This concentration reached 10-fold and 3-fold values at 32 and 34 HAI respectively. At 24 hours after inoculation, 10% of the selected leaves showed mild symptoms while 20% of the selected leaves showed mild symptoms at 48 hours after inoculation. These results indicate that methyl salicylate might alert a grower of the presence of a *B. cinerea* infection of tomato plants at greenhouse scale.

In CHAPTER 6, a model is described to calculate the concentrations of plant volatiles in a greenhouse on the basis of source and sink behaviour of these volatiles. This model was used to determine whether volatiles can be used as an indicator of the presence of a *B. cinerea* infection in a large-scale tomato production greenhouse with a volume of 5×10^4 m^3 containing 2.2×10^4 plants. Seven experiments were done to parameterise the model for the three trichome damage-induced VOCs α-pinene, α-terpinene, and β-caryophyllene, for the lipoxygenase product (*Z*)-3-hexenol) and for the volatile phytohormone methyl salicylate. Four scenarios were considered to predict the effect of the fraction of infected plants and the effect of the air exchange rate on the *B. cinerea*-induced increase in concentration of these VOCs. The precision and detection limits of GC coupled to MS or FID were compared with the *B. cinerea*-induced increase in concentration of the previous mentioned VOCs to determine the appropriateness of these instruments for measuring the increase. Based on model predictions, the *B. cinerea*-induced increase in concentration of methyl salicylate is detectable in a large-scale tomato production greenhouse when at least three conditions are met: (a) windows are opened, (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 1 h, and (c) 5% of the plants are infected. The *B. cinerea*-induced increase in concentration of methyl salicylate is also detectable when (a) windows are closed, (b) the *B. cinerea*-induced increase in emission of methyl salicylate continues for at least 6 h, and (c) 5% of the plants are infected. The increase in concentration of α-pinene, α-terpinene and β-caryophyllene cannot be measured with the above mentioned analytical instruments. The *B. cinerea*-induced increase in concentration of (*Z*)-3-hexenol is detectable under all scenarios. However, it is expected that besides infected plants, many additional sources of lipoxygenase products exist including plant debris and nearby field crops especially upon harvest or stress. Plant debris is nearly always present in greenhouses, and harvest and/or stress of nearby crops is extremely difficult to predict. Therefore, it is

recommended to focus on the detection of methyl salicylate to indicate *B. cinerea* infections in large-scale tomato production greenhouses.

In CHAPTER 7, attention is paid to the automatic processing of data. Almost all of the analyses described in this thesis were conducted using GC-MS. The data from this instrument is relatively complex and experienced analysts are required to process the data. This makes the use of this instrument costly and therefore reduces its widespread application. Developments in computer hardware and software have increased the opportunity to automatically process GC-MS data. The results in CHAPTER 7 show that the concentrations of VOCs inside a greenhouse can be determined automatically.

In CHAPTER 8, the answers to the main research questions are provided and the strengths and limitations of VOC based crop monitoring are discussed. A limitation of this method is the lack of specificity; as mentioned before, a *B. cinerea* specific detection would be feasible if an infection consistently results in the emissions of unique VOCs.

The emission of large amounts of unique substances from tomato upon *B. cinerea* induced stress seems unlikely since this research, and literature show that emissions of many of the same substances, including all VOCs detected in our experiments, increase upon many other biotic and abiotic stresses of tomato. A unique time course of *B. cinerea*-induced emissions of VOCs may also be sufficient to allow a *B. cinerea* specific detection. However, this research, and literature, do not provide any example of such a unique time course. Therefore, it is improbable that an infection with *B. cinerea* can be specifically detected based on the time course of stress-induced volatile emissions only.

But, this research provides some evidence that VOCs can be used as an early warning sign for growers to indicate crop stress of which the presence of a *B. cinerea* infection is an option. The phytohormone methyl salicylate is then an important candidate since the concentration of this VOC did not increase after general crop operations while it did increase after infection with *B. cinerea*. The detection of lipoxygenase products seems not suitable for that since these VOCs are also emitted from nearby field crops, especially upon harvest, and probably also from plant debris and during pruning. Also monoterpenes and most sesquiterpenes are not suitable since their increase upon infection is probably too low to be detectable. Furthermore, these type of compounds are stored in glandular trichomes of tomato and their emissions

increase upon shoot removal, fruit picking and almost certainly all other crop operations, and upon increases in temperature. An exception is α-copaene; this VOC is not stored in large amounts in trichomes and emissions of this compound increased from detached leaves upon infection. However, the emission of α-copaene did not increase from intact plants upon infection and seems therefore not suitable.

Ultimately, an increase in the concentration of methyl salicylate might serve as an effective warning sign for the presence of *B. cinerea* since the diversity of stress factors that occur in a tomato production greenhouse is often limited. At this moment, GC-MS and GC-FID instruments are expensive which seems the most important reason to cite against the use of GC-MS or GC-FID for cop monitoring through plant-emitted VOCs. An important point for future research is the development of an affordable instrument for gas analysis, specifically designed for application in horticulture practice.

SAMENVATTING

De teelt in kassen kenmerkt zich door monocultuur wat betekent dat vaak één soort gewas wordt geteeld. Planten staan doorgaans dicht op elkaar en omdat het klimaat regelbaar is, kan desgewenst jaarrond worden geteeld. Het jaarrond aanwezig zijn van één gewassoort bij een hoge plantdichtheid is risicovol omdat dit uitbraak en verdere verspreiding van ziekte stimuleert. Om uitbraak en verspreiding tegen te gaan wordt gebruik gemaakt van bestrijdingsmiddelen. Echter, met het oog op voedselveiligheid is juist een reductie in het gebruik van deze middelen noodzakelijk. Verder zijn bestrijdingsmiddelen kostbaar, ongezond voor de medewerkers in de kas en slecht voor het milieu.

Om bestrijdingsmiddel te besparen wordt het gewas regelmatig geïnspecteerd op symptomen van ziekte. Als deze symptomen tijdig worden opgespoord kan er lokaal en met minder middel worden gewerkt. Deze inspecties zijn belangrijk, met name in grootschalige kassen omdat ziektes hier eenvoudig over een groot oppervlak kunnen verspreiden. Ze zijn ook kostbaar omdat veel tijd en geschoold personeel noodzakelijk zijn. Daardoor is de wens ontstaan om automatisch zieke planten op te kunnen sporen, liefst in een zeer vroeg stadium. Een mogelijk principe is het meten van vluchtige stoffen afgescheiden door planten. Dit principe is gebaseerd op het gegeven dat de afscheiding van deze stoffen wijzigt bij ziekte. Dit biedt de mogelijkheid om vluchtige stoffen te gebruiken om ziekte te kunnen detecteren.

Het doel van het in dit proefschrift beschreven onderzoek was om te verkennen of vluchtige stoffen afgescheiden door planten bruikbaar zijn om plantenziekte te detecteren in een grootschalige kas. Specifiek hebben we ons gericht op de detectie van *Botrytis cinerea* infecties in tomaat (*Lycopersicon esculentum*). Er werd gekozen voor *B. cinerea* omdat deze ziekteverwekker aanzienlijke schade aanricht in kassen, niet alleen in tomaat maar ook in andere gewassoorten. Tomaat werd geselecteerd omdat de kasteelt van dit gewas wereldwijd een belangrijke economische activiteit is.

Om te kunnen bepalen of op basis van vluchtige stoffen *B. cinerea* infectie kan worden gedetecteerd, is kennis vereist over afscheiding van vluchtige stoffen door gezonde en *B. cinerea* geïnfecteerde planten. Een *B. cinerea* specifieke detectie is mogelijk als een infectie consequent leidt tot de emissie van unieke stoffen. Om de uniekheid te kunnen

bepalen werd gekeken naar factoren welke mogelijk een effect hebben op de emissies van tomatenplanten zoals droogte, maar ook een toename van temperatuur of lichtintensiteit en gewashandelingen zoals oogst. Deze factoren zijn in overweging genomen om de betrouwbaarheid van de detectie op basis van vluchtige stoffen te kunnen inschatten. Een belangrijk aspect is het kennen van de door *B. cinerea* geïnduceerde concentraties van vluchtige stoffen in een grootschalige kas. Deze concentraties zijn immers leidend bij de evaluatie van sensoren welke deze concentraties kunnen meten in een kas.

In het in dit proefschrift beschreven onderzoek worden onderstaande onderzoeksvragen behandeld.

1. Wat is het effect van een *B. cinerea* infectie op de emissies van vluchtige stoffen door tomatenplanten?
2. Zijn de door *B. cinerea* geïnduceerde emissies van vluchtige stoffen door tomatenplanten specifiek voor een infectie met deze ziekteverwekker?
3. Zijn de door *B. cinerea* geïnduceerde concentraties van vluchtige stoffen detecteerbaar in een grootschalige tomaat producerende kas?

In de beginfase van het onderzoek is gekeken naar de emissies van vluchtige stoffen door afgesneden tomatenbladeren welke individueel werden opgesloten in Petri schalen (HOOFDSTUK 2). De vluchtige stoffen werden statisch en dynamisch geconcentreerd tijdens het bemonsteren van de lucht in deze schalen. Lucht rondom niet geïnfecteerde en *B. cinerea* geïnfecteerde bladeren werd op deze wijze bemonsterd. De monsters werden geanalyseerd doormiddel van gaschromatografie (GC) gevolgd door massaspectrometrie (MS) of vlamionisatie detectie (FID) om zo inzicht te verkrijgen in emissies van niet geïnfecteerde en *B. cinerea* geïnfecteerde tomatenplanten. Afgesneden bladeren vertoonden na infectie verhoogde emissies van het sesquiterpeen α-copaeen.

In vervolgexperimenten werden emissies van intacte tomatenplanten onderzocht (HOOFDSTUK 3). Per experiment werden een aantal niet geïnfecteerde of *B. cinerea* geïnfecteerde planten gedurende drie dagen opgesloten in een kamer met conditionering van temperatuur, luchtvochtigheid, lichtintensiteit, en CO_2. In deze kamer werd gefilterde lucht ingeblazen en

de uitgaande lucht werd ieder uur dynamisch bemonsterd, waarna dit monster direct werd geanalyseerd met behulp van GC-MS. Hierbij werden tenminste 40 door tomatenplanten afgescheiden vluchtige stoffen geïdentificeerd. De vijftien meest dominante stoffen werden met behulp van standaarden en gemeten bladoppervlakten genormaliseerd naar emissies per eenheid oppervlak en tijd. Deze fluxdichtheden vertoonden grote variaties tussen herhaling, zowel bij niet geïnfecteerde als bij *B. cinerea* geïnfecteerde planten.

In tegenstelling tot experimenten met afgesneden *B. cinerea* geïnfecteerde bladeren werden alcoholen en aldehyden aangetroffen in de experimenten met *B. cinerea* geïnfecteerde planten. Deze stoffen worden aangeduid als lipoxygenase producten; ze ontstaan door een enzymatische peroxidatie onder invloed van lipoxygenasen. Deze stoffen worden afgescheiden door beschadigde planten waarbij schade aan celmembranen een belangrijke rol speelt. Meest dominant was het lipoxygenase product (*Z*)-3-hexenol met gemeten flux dichtheden tot 0.5 nmol m^{-2} s^{-1}. De emissie van lipoxygenase producten werd vermoedelijk veroorzaakt door *B. cinerea* geïnduceerde schade aan celmembranen. Emissie van deze stoffen waren hoger en begonnen eerder bij zware aantasting in vergelijking met milde aantasting. De toename in de emissies van lipoxygenase producten vonden plaats enkele uren na inoculatie. Dit geeft aan dat deze stoffen een vroege indicatie van celmembraanschade kunnen vormen, nog voordat symptomen van een *B. cinerea* infectie zichtbaar zijn.

Zowel niet geïnfecteerde als *B. cinerea* geïnfecteerde planten vertoonden emissies van monoterpenen en sesquiterpenen waaronder *α*-copaeen. Echter, in tegenstelling tot afgesneden bladeren, was de emissie van *α*-copaeen bij geïnfecteerde planten niet significant hoger. Dit verschil kan worden verklaard door het gebruik van intacte planten in plaats van afgesneden bladeren. Het dag/nacht ritme in de flux dichtheden van deze stoffen werd vooral toegewezen aan verschillen in bladtemperatuur. Verhoogde fluxdichtheden van monoterpenen en sesquiterpenen werden aangetroffen bij zwaar geïnfecteerde planten tot maximaal 1 nmol m^{-2} s^{-1}. De verhouding in fluxdichtheden tussen de monoterpenen onderling bleef echter gelijk na infectie wat waarschijnlijk duidt op een generiek mechanisme: schade aan glandulaire trichomen.

Niet geïnfecteerde en *B. cinerea* geïnfecteerde planten vertoonden emissies van methylsalicylaat en (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). Het dag/nacht

ritme in de flux dichtheden van deze stoffen kan worden toegewezen aan verschillen in lichtintensiteit. Bij niet geïnfecteerde planten namen de flux dichtheden van methylsalicylaat en TMTT langzaam toe gedurende het experiment, waarschijnlijk als gevolg van stress door insluiten van planten. Bij *B. cinerea* geïnfecteerde planten namen de flux dichtheden van methylsalicylaat en TMTT sneller toe gedurende het experiment in nmol m^{-2} s^{-1} orde van grootte. Er kon geen duidelijk verband worden vastgesteld tussen de infectiegraad en de toename in flux dichtheden van methylsalicylaat en TMTT. Deze stoffen worden aangeduid als hormoonstoffen waarvan emissies bij vele plantsoorten toeneemt als gevolg van biotische en abiotische stress.

In HOOFDSTUK 4 staan experimenten beschreven welke zijn uitgevoerd in een kleine proefkas met 60 tomatenplanten op een vloeroppervlak van 40 m^2. Deze planten werden gedurende twee maanden wekelijks op een kunstmatige manier beschadigd om zo een *B. cinerea* infectie te simuleren. Deze beschadiging bestond uit het strijken van de stengels met het doel glandulaire trichomen te beschadigen en het verwijderen van zijscheuten om zo celmembranen te beschadigen. Daarnaast werd gekeken naar het effect van vruchten plukken op de concentraties van vluchtige stoffen afgescheiden door de planten in de kas. Luchtmonsters werden op drie plaatsen in de kas verzameld en vervolgens naar het laboratorium getransporteerd voor analyse met behulp van GC-MS. De concentraties van vluchtige stoffen was altijd vrijwel gelijk tussen de drie plaatsen wat werd verklaard door de hoge interne luchtcirculatie van 2×10^4 m^3 lucht per uur.

In monsters verkregen voor beschadiging van de planten werden ten minste negen monoterpenen, vier sesquiterpenen, drie fenolen en één homoterpeen aangetroffen. Deze stoffen werden ook in de voorgaande laboratoriumexperimenten aangetroffen. Het monoterpeen *β*-phellandreen was altijd in de hoogste concentratie aanwezig. Bij een gewasleeftijd van zeven weken was de concentratie van deze stof circa 0.06 nmol per mol lucht. Bij een gewasleeftijd van twaalf weken was de concentratie van deze stof opgelopen tot circa 0.14 nmol per mol lucht. Het strijken van de stengels, het verwijderen van zijscheuten en het plukken van vruchten resulteerden in een toename in de concentraties van alle monoterpenen en de meeste sesquiterpenen met maximaal een factor 60. Een dergelijke toename was verwacht omdat trichomen van tomatenplanten monoterpenen en sesquiterpenen bevatten. De verhouding in concentraties tussen de monoterpenen *β*-phellandreen en 2-careen

bleef gelijk na het strijken van de stengels terwijl de verhouding in concentraties tussen het monoterpeen *β*-phellandreen en het sesquiterpeen *β*-caryofylleen wijzigde. Dit biedt de mogelijkheid om op basis van verhoudingen tussen individuele vluchtige stoffen schade aan glandulaire trichomen te detecteren.

In tegenstelling tot een infectie met *B. cinerea* bleven zowel na het strijken van de stengels, na het verwijderen van zijscheuten en na het plukken van vruchten de concentraties van *α*-copaeen, methylsalicylaat en TMTT stabiel. Hiervoor werden drie redenen aangedragen. Ten eerste, eventueel van *B. cinerea* afkomstige signaalstoffen zijn dan afwezig. Dergelijke signaalstoffen spelen mogelijk een belangrijk rol bij emissies van vluchtige stoffen door planten. Ten tweede, er was telkens sprake van een momentaan type schade terwijl een *B. cinerea* infectie wellicht schade met een meer continue karakter veroorzaakt. Er zijn aanwijzingen dat het tijdsverloop van de schade invloed heeft op de emissie van vluchtige stoffen door planten. Ten derde werd er direct na de schade gemeten. Er zijn verschillende voorbeelden bekend uit de literatuur waarbij de emissies van bepaalde vluchtige stoffen, waaronder methylsalicylaat, pas enkele uren na aanvang van stress toenamen.

In tegenstelling tot het strijken van stengels en het plukken van vruchten zorgden het verwijderen van zijscheuten voor een detectie van het lipoxygenase product (*Z*)-3-hexenol in concentraties van acht tot twintig pmol per mol lucht. Deze stof duidt op cel membraan schade en het is dus mogelijk om dit type schade te detecteren in een kleinschalige kas.

Het experiment beschreven in HOOFDSTUK 5 is uitgevoerd in dezelfde proefkas als de kas beschreven in HOOFDSTUK 4. Nu werden alle 60 planten tegelijkertijd kunstmatig geïnoculeerd met een sporen-suspensie van *B. cinerea*. Het doel was het effect van inoculatie te bepalen op concentraties van de typische cel membraan schade stoffen (lipoxygenase producten), op concentraties van de typische trichoom schade stoffen (twaalf monoterpenen en een viertal sesquiterpenen), en op de concentraties van de hormoonstoffen methylsalicylaat en TMTT. Direct na inoculatie werd de kaslucht met een tijdsinterval van 1 uur bemonsterd tot 72 uur na inoculatie. Deze monsters werden naar het laboratorium getransporteerd voor GC-MS analyse. Tien bladeren werden willekeurig geselecteerd om zichtbare symptomen van *B. cinerea*-infectie te volgen.

Lipoxygenase producten werden niet gedetecteerd na inoculatie. Dit werd verklaard door het minimaal optreden van *B. cinerea* symptomen en/of een mogelijk acute verwijdering van lipoxygenase producten als gevolg van opname door in de kas aanwezig condenswater. De concentraties van monoterpenen, de meeste sesquiterpenen, en TMTT bleef stabiel na inoculatie terwijl de concentratie van α-copaeen fluctueerden volgens het dag/nacht ritme. Deze fluctuatie werd wellicht veroorzaakt door de lichtafhankelijke synthese van α-copaeen wat aangeeft dat, in een kleinschalige kas, vluchtige stoffen gebruikt kunnen worden om de biosynthese van stoffen in het gewas te meten op niet invasieve wijze. De concentratie van methylsalicylaat na inoculatie was twintig picomol per mol lucht. Deze concentratie nam toe met een factor 10 en een factor 3 op respectievelijk 32 en 34 uur na inoculatie. Op respectievelijk 24 en 40 uur na inoculatie vertoonden 10% en 20% van de geselecteerde bladeren milde symptomen. Deze resultaten geven een indicatie dat methylsalicylaat mogelijk gebruikt kan worden om telers te waarschuwen voor de aanwezigheid van een *B. cinerea* infectie in de kas.

HOOFDSTUK 6 beschrijft een model waarmee op basis van aanvoer en afvoer, de concentraties van door planten afgescheiden vluchtige stoffen in een grootschalige kas is berekend. Met dit model werd bepaald of deze vluchtige stoffen gebruikt kunnen worden als indicator voor de aanwezigheid van een *B. cinerea* infectie in dergelijke kas. Een zevental experimenten werd uitgevoerd om het model te parameteriseren voor de drie trichoom schade stoffen α-pineen, α-terpineen en β-caryofylleen, voor het lipoxygenase product (*Z*)-3-hexenol, en voor de hormoonstof methylsalicylaat. Vier scenario's werden doorgerekend om het effect van ventilatie en het percentage geïnfecteerde planten op de door *B. cinerea*-geïnduceerde toename in concentratie van voorgenoemde stoffen te voorspellen. Doormiddel van omschreven precisie en detectie limieten van GC gekoppeld aan MS of FID werd bepaald of deze toename daadwerkelijk detecteerbaar is.

Op basis van model gebaseerde voorspellingen blijkt dat de *B. cinerea*-geïnduceerde toename in concentratie van methylsalicylaat detecteerbaar is in een grootschalige kas onder ten minste drie voorwaarden: (a) de ventilatieramen open, en (b) de *B. cinerea*-geïnduceerde toename in de emissie van methylsalicylaat gedurende tenminste 1 uur plaatsvindt, en (c) 5% van de planten geïnfecteerd is. De *B. cinerea*-geïnduceerde toename in concentratie van methylsalicylaat is bovendien detecteerbaar indien (a) de ventilatieramen gesloten zijn, en (b) de *B. cinerea*-geïnduceerde toename in de emissie van methylsalicylaat gedurende tenminste 6 uur plaatsvindt, en (c) 5% van de planten geïnfecteerd is.

De toename in concentraties van *α*-pineen, *α*-terpineen en *β*-caryofylleen zijn niet detecteerbaar met bovengenoemde instrumenten. De *B. cinerea*-geïnduceerde toename in concentratie van het lipoxygenase product (*Z*)-3-hexenol is detecteerbaar voor alle vier de scenario's. Echter, naast geïnfecteerde planten zijn er vermoedelijk talloze andere bronnen van lipoxygenase producten zoals bladafval en nabijgelegen veldgewassen, met name tijdens oogst of stress van dit veldgewas. Bladafval is vrijwel altijd aanwezig in kassen en oogst of stress van nabijgelegen veldgewassen in zeer moeilijk te voorspellen. Het is daarom aan te bevelen om te richten op de detectie van methylsalicylaat als indicator voor aanwezigheid van *B. cinerea* infecties in grootschalige tomaten producerende kassen.

HOOFDSTUK 7 behandelt het automatisch verwerken van data. Vrijwel alle in dit proefschrift beschreven analyse zijn uitgevoerd met GC-MS. De data afkomstig van dit instrument is relatief complex en voor verwerking van deze data zijn ervaren analisten noodzakelijk. Dit maakt de inzet van dit instrument kostbaar en werkt dus beperkend. Ontwikkelingen op het gebied van computerhardware en -software bieden mogelijkheden om GC-MS data automatisch te verwerken. De resultaten in HOOFDSTUK 7 laten zien dat automatisch de concentraties van vluchtige stoffen kan worden gevolgd in een kas.

In HOOFDSTUK 8 wordt getracht antwoord te geven op de hoofd onderzoeksvragen. Tevens worden de sterke en zwakke punten van het op vluchtige stoffen gebaseerd monitoren van gewas bediscussieerd. Een zwak punt van deze methode is het gebrek aan specificiteit; zoals eerder gezegd is een *B. cinerea* specifieke detectie mogelijk als een infectie consequent leidt tot de emissie van unieke stoffen.

Emissie van grote hoeveelheden specifieke stoffen door tomaat na infectie met *B. cinerea* is onwaarschijnlijk omdat uit dit onderzoek en uit literatuur blijkt dat emissies van veelal dezelfde stoffen, inclusief de door ons gevonden stoffen, toenemen na toediening van andere biotische en abiotische stressoren. Een uniek tijdsverloop van *B. cinerea* geïnduceerde emissies van vluchtige stoffen biedt een alternatief voor *B. cinerea* specifieke detectie. Echter, dit onderzoek, maar ook literatuur geeft geen voorbeelden van dergelijk uniek tijdsverloop en het lijkt dus onwaarschijnlijk dat het tijdsverloop gebruikt kan worden om een *B. cinerea* specifieke infectie te detecteren.

Dit onderzoek geeft aan dat vluchtige stoffen wel gebruikt kunnen worden om gewasstress te detecteren in een kas, waarbij een *B. cinerea* infectie de aanleiding kan zijn. De hormoonstof methylsalicylaat lijkt hiervoor het meest geschikt omdat de concentratie van deze stof gelijk bleef na gewashandelingen terwijl de concentratie toenam na infectie met *B. cinerea*. De detectie van lipoxygenase producten lijkt hiervoor ongeschikt omdat naast geïnfecteerde planten er vermoedelijk talloze andere bronnen van lipoxygenase producten zoals bladafval en nabij gelegen veldgewassen, met name tijdens oogst of stress van dit veldgewas. Bladafval is vrijwel altijd aanwezig in kassen en oogst of stress van veldgewassen in zeer moeilijk te voorspellen.

Ook monoterpenen en sesquiterpenen zijn ongeschikt omdat de toename in concentratie na infectie waarschijnlijk onvoldoende is. Daarnaast is dit type stoffen opgeslagen in glandulaire trichomen van tomaat waardoor concentraties toenemen tijdens schade aan deze trichomen door bijvoorbeeld het verwijderen van zijscheuten, het plukken van vruchten en waarschijnlijk iedere andere gewashandeling. Een uitzondering hierop is α-copaeen; deze stof is niet in grote hoeveelheid opgeslagen in trichomen en de concentratie van deze stof lijkt toe te nemen na *B. cinerea* infectie van losse bladeren. Echter, de emissie van deze stof nam niet toe na infectie van intacte planten en lijkt dus ongeschikt.

Uiteindelijk geeft een toename in de concentratie van methylsalicylaat mogelijk voldoende aanwijzing voor aanwezigheid van een *B. cinerea* infectie in een kas omdat de diversiteit van stressfactoren in een tomaten producerende kas gering is. De aanschafprijs van gas analyse instrumenten is echter te hoog om deze in te zetten voor gewasmonitoring op basis van vluchtige stoffen welke door planten worden afgescheiden. Een belangrijk punt voor verder

onderzoek is dan ook de ontwikkeling van goedkopere gasanalyse instrumenten, speciaal ontworpen voor toepassing in de glastuinbouwpraktijk.

CURRICULUM VITAE

Rudolf Martinus Cornelis (Roel) Jansen werd geboren op 9 november 1976 te Boxmeer. Na het behalen van zijn Havo diploma aan het Elzendaal College te Boxmeer in 1996 begon hij in hetzelfde jaar met de studie werktuigbouwkunde aan de Hogere Technische School te Arnhem. Gedurende deze studie kwam hij in aanraking met de integratie van techniek en landbouwkundige aspecten. Tijdens deze studie heeft hij een stage voltooid bij het Magoye landbouwkundig onderzoeksstation, Zambia. In 2000 begon hij aan de studie Landbouwtechnische Wetenschappen aan de Wageningen Universiteit. Hier studeerde hij in 2003 af met een afstudeervak bij de leerstoelgroep Agrarische Bedrijfstechnologie. In januari 2004 begon hij aan zijn promotieonderzoek bij deze leerstoelgroep, waar hij aan het onderwerp van dit proefschrift heeft gewerkt. Vanaf maart 2009, is hij werkzaam als onderzoeker bij Wageningen UR Glastuinbouw waar hij met name betrokken zal zijn bij tuinbouwtechnologie.

CURRICULUM VITAE

Rudolf Martinus Cornelis (Roel) Jansen was born on November 9th, 1976 in Boxmeer, The Netherlands. He completed his secondary school in 1996 at the Elzendaal College in Boxmeer. From 1996 until 2000 he studied Mechanical Engineering at the HAN University of Applied Sciences. During this study he did an internship for 7 months at the Magoye Agricultural Research Station, Zambia. From 2000 until 2003 he studied Agricultural Engineering at the Wageningen University. He completed this study with a major thesis at the Farm Technology Group. Between 2004 and 2009 he was employed by this group where he worked on his PhD research. Results of his research during this time are illustrated in this thesis. Since March 2009 the author works as a researcher at Wageningen UR Greenhouse Horticulture.

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Magazine articles

Jansen, R.M.C., 2003. Vroegtijdig Phytophthora opsporen met biosensor. Landbouwmechanisatie, 54(6): 15.

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Jansen, R.M.C., 2007. Machinale Botrytis detectie. Glastuinbouwtechniek magazine, 3: 22-25.

Jansen, R.M.C., 2007. Sniffing out disease. Precision Ag News, 4 (1): 20.

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PE&RC PHD EDUCATION CERTIFICATE

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (4.2 ECTS)

- Plant volatile analysis and the monitoring of plant health – a review. Published in the journal 'Environmental Control in Biology' 47(2),21-34 (2009)

Writing of Project Proposal (3 ECTS)

- Three ESF (European Science Foundation) proposals granted (2006 & 2007)

Laboratory Training and Working Visits (5.3 ECTS)

- Plant volatile analysis-working visit; Research Centre Jülich, Germany (2006 and 2007)
- Thermal desorption-laboratory training; Interscience B.V., Breda, the Netherlands (2007)

Post-Graduate Courses (2.8 ECTS)

- Basic statistics; PE&RC (2005)
- Advanced statistics; PE&RC (2005)

Deficiency, Refresh, Brush-up Courses (3.4 ECTS)

- Organic chemistry; WUR (2004)
- Thesis supervision; WUR (2005)
- NWO Course-write it right; NWO (2005)
- Basic course didactics; WUR (2008)

Competence Strengthening / Skills Courses (7.3 ECTS)

- Time planning and project management; PE&RC (2004)
- Scientific publishing; PE&RC (2005)
- Personal assessment; PE&RC (2005)
- Academic writing; PE&RC (2006)
- Scientific writing; PE&RC (2006)
- Technology assessment; Rathenau Institute (2006)
- The art of writing; PE&RC (2007)

Discussion Groups / Local Seminars and Other Scientific Meetings (4.2 ECTS)

- FRONTIS Workshop; Chemical Ecology, Wageningen, the Netherlands (2005)
- ESF-VOCBAS Workshop; Plant VOC, Jülich, Germany (2006)
- Plant Volatile analysis meetings (2006)
- ESF-VOCBAS Workshop; Plant VOC, Wageningen, the Netherlands (2006 and 2008)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (2.1 ECTS)

- Introduction PE&RC weekend; (2005)
- PE&RC day (2005, 2006, 2007 and 2008)

International Symposia, Workshops and Conferences (7.25 ECTS)

- BCPC International conference; Glasgow, UK (2005)
- APS-CPS-MSA Joint annual meeting; Quebec, Canada (2006)
- PR-IR International joint workshop; Doorn, the Netherlands (2007)
- Botrytis werkgroep bespreking; the Netherlands (2007)
- Greensys international symposium; Napels, Italy (2007)
- European plant biology congress; Tampere, Finland (2008)
- Greensys international symposium; Quebec, Canada (2009)

Courses in Which the PhD Candidate Has Worked as a Teacher

- Problem exploration agrotechnology; Farm Technology Group; 5 days

Supervision of MSc Students

- Biosensing-Plant volatile analysis; 4 students; 40 days