Health Monitoring of Plants by Their Emitted Volatiles: a Temporary Increase in the Concentration of Methyl Salicylate after Pathogen Inoculation of Tomato Plants at Greenhouse Scale

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Abstract

This paper describes a method to alert growers of the presence of a pathogen infection in their greenhouse based on the detection of pathogen-induced emissions of volatile organic compounds (VOCs) from plants. Greenhouse-grown plants were inoculated with spores of a fungus to learn more about this concept. The specific objective of the present study was to determine whether VOCs are detectable after inoculation, and if so, to determine the time course of the concentrations of these compounds. To achieve this objective, we inoculated 60 greenhouse-grown tomato plants (*Lycopersicon esculentum*) with an aqueous suspension of *Botrytis cinerea* spores. Upon inoculation, the greenhouse air was sampled semi-continuously with a one hour time interval until 72 hours after inoculation (HAI). The samples were transferred to the laboratory and analysed using gas chromatography - mass spectrometry. 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 HAI. Results demonstrated no detection of C_6-compounds, and an almost constant concentration of all monoterpenes, most sesquiterpenes, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. However, the concentration of methyl salicylate increased 10-fold and 3-fold at 32 and 34 HAI respectively. At 24 HAI, 10% of the selected leaves showed mild symptoms while 20% of the selected leaves showed mild symptoms at 48 HAI. These results indicate that methyl salicylate might alert a grower of the presence of a *B. cinerea* infection of tomato plants at greenhouse scale. Further research is required to confirm these findings.

INTRODUCTION

In spite of tremendous efforts to manage plant diseases, phytopathogens still cause serious economic damage in greenhouse cultivation (Elad, 1999). Early detection of pathogen infections would enable better management and control. Although crops are subjected to regular inspections to look for symptoms, diseases are often overlooked mainly because 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

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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. (2009b) 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., 2009a). In the latter study, C_6-compounds 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. (2009b) also reported an increase in the concentration of several monoterpenes 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., 2009a) 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. (2009b) 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. '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 23 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 22/16±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 have been described elsewhere (Jansen et al., 2009a).

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. 1A and B). The time course of the temperature inside of the greenhouse was similar in between those three days (Fig. 1C).

Monitoring of Visual Symptoms

Two pictures, both taken at 72 HAI are provided in Figure 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". Figure 3 summarizes

these classification results.

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 2 HAI and then decreased to a nearly constant level. As a representative of the monoterpenes, Figure 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 2 HAI and then decreased to a nearly constant level. As a representative of the sesquiterpenes, Figure 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. 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. 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).

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. 1B and C), 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., 2009b), C_6-compounds were undetected in samples collected in the greenhouse. A first explanation may concern the low extent of damage to cell-membranes which was probably not sufficient to induce the release of detectable concentrations of C_6-compounds in greenhouse air. This concept is in agreement with the low percentage of leaves classified as “severe symptoms” and “mild symptoms” throughout the experimental period (Fig. 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 air dryer 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 grown in the greenhouse. The relatively high concentrations of all mono- and most sesquiterpenes at 2 HAI (Fig. 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 glandular 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 mono- 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 mono- and most sesquiterpenes might be the relatively low amount of air exchange compared to the laboratory setup described in Jansen et al. (2009b); 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 was 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 glandular 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 sesquiterpenes 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 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. 1 with Fig. 4), suggesting that 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., 2009b), 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., Dicke et al., 1998; Deng et al., 2004). 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., 2009a). 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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Figures

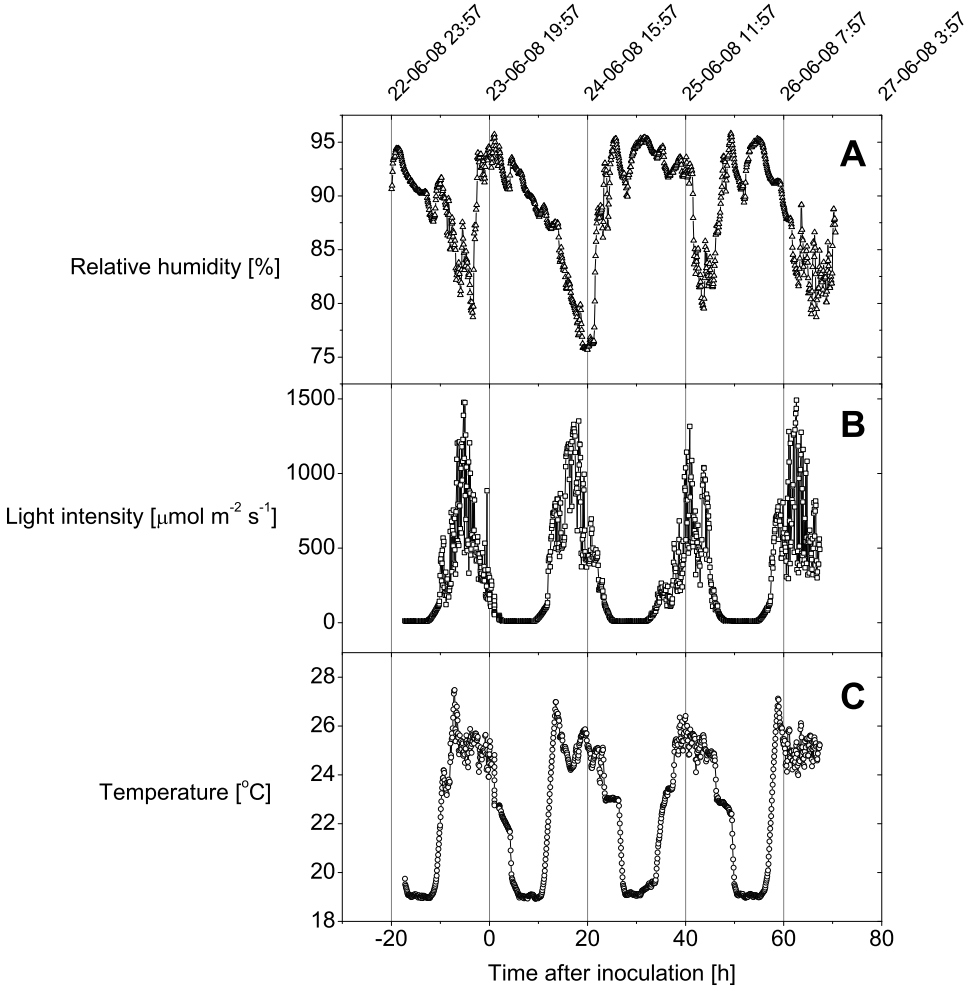

Fig. 1. The time course of (A) the relative humidity (-Δ-), (B) light intensity (-□-), and (C) temperature (-○-) inside the greenhouse.

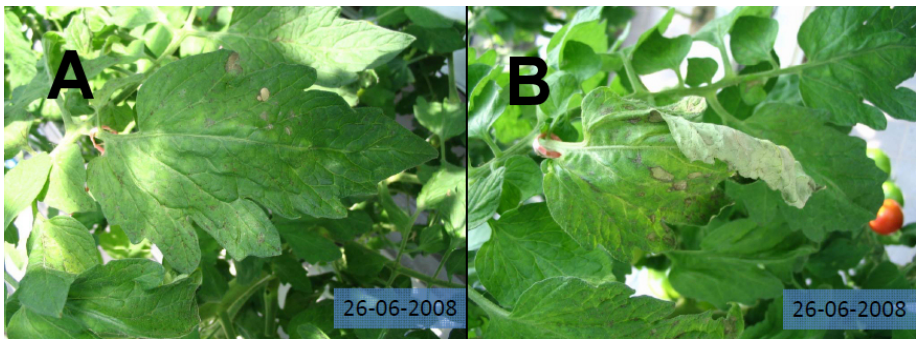

Fig. 2. Pictures of leaves classified as (A) "mild symptoms", and (B) "severe symptoms". Both pictures were recorded at 72 h after inoculation.

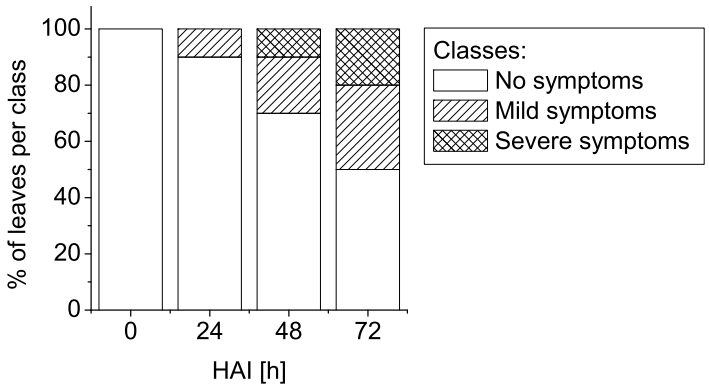

Fig. 3. Classification of ten randomly selected leaves at 0, 24, 48, and 72 h after the inoculation (HAI) of tomato plants with *Botrytis cinerea* spores.

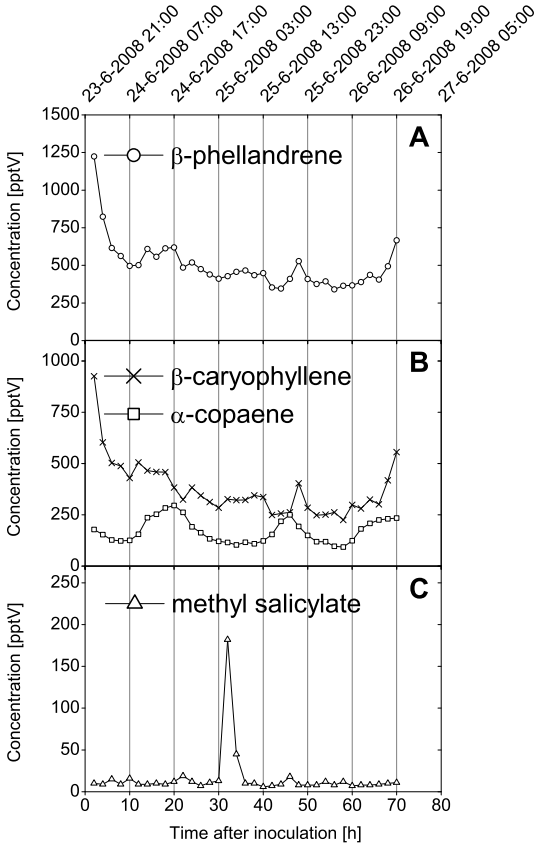

Fig. 4. The time course of the concentration of (A) the monoterpene *β*-phellandrene (-○-), (B) the sesquiterpenes *β*-caryophyllene (-×-) and *α*-copaene (-□-), and (C) methyl salicylate (-Δ-) after the inoculation of tomato plants with *Botrytis cinerea* spores.