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Use of Biogenic Volatile Organic Compounds to assess the Health Status of Tomato Plants (*Solanum Lycopersicum*) cv. "Moneymaker"

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ABSTRACT

The goal of the present study is to test if tomato biogenic volatile organic compounds (BVOC) can be used as an early stress indicator. In this study, influences of drought and biotic stresses on BVOC emissions were investigated under controlled conditions. The BVOC under study were constitutively emitted monoterpenes (MT), stress-induced terpenoids (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), (*E*)-β-ocimene, α-copaene as well as stress induced green leaf volatiles (GLV), hexenyl derivatives (HexD) and methyl salicylate (MeSA).

Under mild drought stress, emissions of TMTT, (*E*)-β-ocimene and HexD increased, but these increases were not attributed directly to drought. Under severe drought, the same emissions decreased almost to zero as a direct consequence of applied drought and transpiration reduction, while emissions of constitutive MT increased due to leaf wilting and trichome damage. The final stage of drought caused membrane damage what resulted in bursts of GLV emissions. None of these effects is restricted to drought.

The second part of the study focuses on BVOC emissions from tomato plants exposed to *Botrytis cinerea*, *Oidium neolycopersici, Myzus persicae* and *Trialeurodes vaporariorum*. This study shows that four *de-novo* emissions (α-copaene, (*E*)-β-ocimene, MeSA and HexD) were associated directly to plants reaction to the biotic stresses. Experimental results indicate that *Botrytis cinerea* infected plants had predominantly jasmonic pathway activated and *Myzus persicae / Trialeurodes vaporariorum* infested plants had predominantly salicylic pathway activated. In plants infected with *Oidium neolycopersici*, BVOC emission were very low hampering identification of a pathway activated by the stress.

Compounds induced by biotic stress were studied to assess the usability of such emissions for biotic stress detection in greenhouses. Four target compounds were chosen for biotic stress detection in tomato greenhouses:α-copaene, MeSA, HexD and GLV.

KURZFASSUNG

Das Ziel dieser Studie ist zu ermitteln, ob die Emissionen biogener flüchtiger organischer Verbindungen (BVOC) aus Tomaten als Indikator zur frühen Stresserkennung geeignet sind. In dieser Studie wurden die BVOC Emissionen aus Tomate als Folge von Trockenstress und als Folge verschiedener biotischer Stressoren unter Laborbedingungen untersucht. Die Emissionen folgender BVOC wurden beobachtet: Monoterpene (MT), stress-induzierten Terpenoide ((*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), (*E*)-β-ocimene, α-copaene), stress-induzierte BVOC aus dem Octadecanoidweg (Green Leaf Volatiles, GLV), Hexenyl Derivate (HexD) und Methylsalicylat (MeSA).

Bei moderatem Trockenstress erhöhten sich die Emissionen von TMTT, (*E*)-β-ocimene und HexD, wobei die Erhöhung der Emissionen nicht in direktem Zusammenhang mit der Trockenheit standen. Bei starker Trockenheit verminderten sich die Emissionen fast bis auf Null. Bei starker Trockenheit erhöhten sich Emissionen der sonst konstitutiv emittierten MT. Verursacht wurde das durch das Welken der Blätter was eine Schädigung der Trichonome und die Erhöhung der MT Emissionen zur Folge hatte. Die beobachteten Erhöhungen der MT und GLV Emissionen sind nicht spezifisch für Trockenstress.

Der zweite Teil der Arbeit beschäftigt sich mit BVOC Emissionen von Tomaten, die *Botrytis cinerea*, *Oidium neolycopersici, Myzus persicae* und *Trialeurodes vaporariorum ausgesetzt waren*. Es konnte gezeigt werden, dass vier *de-novo* Emissionen (α-copaene, (*E*)-β-ocimene, MeSA and HexD) eine direkte Reaktion der Pflanzen auf biotischen Stress waren. Die Ergebnisse weisen darauf hin, dass Pflanzen, die mit *Botrytis cinerea* infiziert waren, hauptsächlich den Jasmonatweg aktiviert hatten. *Myzus persicae* / *Trialeurodes vaporariorum* infizierte Pflanzen hatten hauptsächlich den Salicylatweg aktiviert. Bei Pflanzen, die mit *Oidium neolycopersici* infiziert wurden, waren die Emissionen zu niedrig.

Es wurde untersucht, ob die durch biotischen Stress hervorgerufenen Emissionen zur frühzeitigen Detektion von biotischem Stress in Gewächshäusern geeignet sind. Vier BVOC wurden identifiziert, die hier geeignet sein könnten: α-copaene, MeSA, HexD and GLV.

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LIST OF ABBREVIATIONS

A_{leaf}	leaf area
ATP	adenosine triphosphate
BA	benzoic acid
BVOC	biogenic volatile organic compounds
C_5	unit made of 5 carbon atoms
C_6 GLV	green leaf volatiles made of 6 carbon atoms
cMT	constitutive monoterpene emissions
iMT	induced monoterpene emissions
D/N	day and night light settings
DMAPP	dimethylallyl pyrophosphate
DW	dry weight
ER	endoplasmic reticulum
F_{air}	air flow
FPP	farnesyl pyrophosphate
FW	fresh weight
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
GES	geranyllinalool synthase
GGPP	geranylgeranyl pyrophosphate
GLV	green leaf volatiles
GM	grey mould

GPP	geranyl pyrophosphate
h	hours
HexD	hexenyl derivatives
iMT	induced monoterpene emissions
IPP	isopentenyl pyrophosphate
IR	infrared light
JA	jasmonic acid
JPAC	Jülich Plant Atmosphere Chamber
LOX	lipoxygenase
MeJA	methyl jasmonate
MEP	merthylerythriol phosphate
MeSA	methyl salicylate
MT	monoterpenes
MVA	mevalonate
NADP (NADPH)	nicotinamide adenine dinucleotide phosphate
P450	cytochrome P450 monooxygenase
PL	permanent light
PM	powdery mildew
ppb	parts per billion
PPFD	photosynthetic photon flux density
ppm	parts per million
ppt	parts per trillion
ROS	reactive oxygen species
SAR	systemic acquired resistance

SQT	sesquiterpenes
Tg C	teragrams of carbon
TMTT	(*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
VOC	volatile organic compounds
WF	whitefly
Φ	flux density

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1 GENERAL INTRODUCTION

Volatile organic compounds (VOC) are organic atmospheric trace gases such as hydrocarbons, alcohols, aldehydes, ketones, esters, ethers and acids (Kesselmeier and Staudt, 1999). VOC with biological origin are called biogenic volatile organic compounds (BVOC) and they include several thousand different compounds emitted by plants (Fall*,* 1999). Except from plants, these emissions also originate from other living organisms such as soil bacteria or phytoplankton. Hence, additional sources of BVOC are soils, sediments, freshwater aquatic systems, oceans and animals, but they are emitted in much lower amounts (Fall*,* 1999) than those from vegetation. BVOC annual global emissions in atmosphere are estimated to about 760 Tg C, of which 70 % is isoprene, 11 % are monoterpenes, 6 % methanol, 3 % acetone, 2.5 % sesquiterpenes and other BVOC, each below than 2 % (Sindelarova *et al.,* 2014).

1.1 What are typical tomato emissions?

1.1.1 Tomato constitutive BVOC emissions – biosynthesis, release and ecology

Emissions from non-stressed tomato plants mainly originate from epidermal structures or glandular trichomes located on the surface of the plant. According to Schilmiller *et al*. (2010), trichomes contain stored monoterpenes (MT), including 2-carene, α-phellandrene, α-terpinene, limonene, β-terpinene and β-phellandrene and sesquiterpenes (SQT) including β-caryophyllene, α-humulene and δ-elemene. The dominant BVOC emission from unstressed tomato cultivar Moneymaker is β-phellandrene (Jansen *et al.,* 2009a; Jansen *et al.,* 2011). The amounts of β-phellandrene stored in trichomes reach up to 1000 µg (g DW^{-1} plant material) (Farag and Paré, 2002; Schilmiller *et al.,* 2010).

MT and SQT belong to the group of terpenoids. They are molecules with characteristic C_5 building blocks - isopentenyl pyrophosphate (IPP). MT are made out of two (C_{10}) and SQT out of three (C_{15}) C_5 unites (Ružička, 1953). IPP is a common precursor for all terpenoids. IPP and its isomer, dimethylallyl pyrophosphate (DMAPP), are synthesized in the cytosol via the mevalonate (MVA) pathway or in plastids via the merthylerythriol phosphate (MEP)

pathway. Both pathways for IPP synthesis require ATP, NADPH, and a carbon source such as pyruvate, glyceraldehid-3-phosphate or acetate (Fall, 1999) (Figure 1).

MT are generally considered to be synthesized in plastids and SQT in cytosol (Tholl and Lee, 2011). However, there are reports of cross talk between these two synthetic pathways (Wanke *et al*., 2001). Joining IPP and DMAPP, geranyl pyrophosphate (GPP) is formed, which is the precursor for all MT. Due to different types of monoterpene cyclases, different structural forms of MT are synthetized from GPP (Croteau *et al.,* 1988). Adding another IPP unit to GPP, farnesyl pyrophosphate (FPP) is formed. FPP is the precursor for SQT (Tholl and Lee, 2011).

MT and SQT usually have an intensive smell. They are volatile, not very well soluble in water and they are components of plant essential oils (Maffei *et al.,* 2011). In tomato plants, MT and SQT are the origin of the typical "tomato" smell (Kesselmeier and Staudt, 1999) which is necessary for insect-host recognition and plants defence against herbivores (Snyder *et al.,* 1993; Kennedy, 2003). For example, experiments with tomato plants with a low number of trichomes and low constitutive emissions lead to less attractiveness to herbivores and in the same time show higher susceptibility to *Epitrix cucumeris* and *Leptinotarsa decemlineata.* This suggests that MT in tomato plants influence herbivore communication and host selection (Kang *et al.,* 2010). Furthermore, terpenes stored in plant trichomes are toxic to insects with an ability to repel herbivores and to attack predators of herbivores (Peterson *et al.,* 2003; Birkett *et al.,* 2004; Terry *et al.,* 2007; De Moraes *et al.,* 1998; Kessler and Baldwin, 2001).

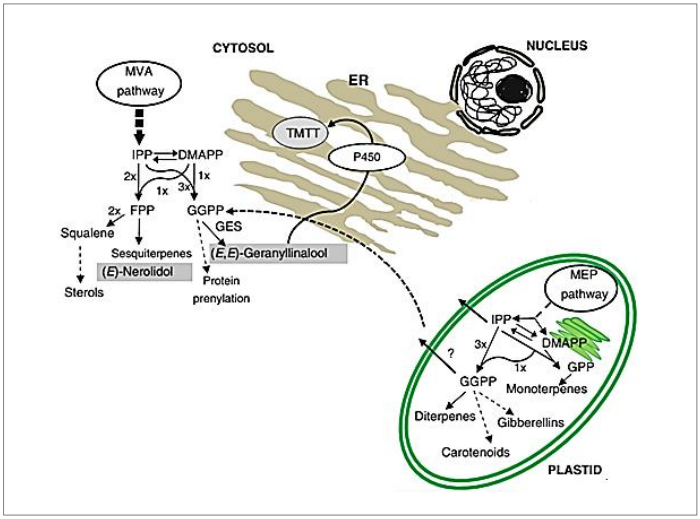


Figure 1 - Simplified presentation of monoterpene, sesquiterpene and homoterpene biosynthesis in the tomato cell. Adjusted from Tholl and Lee (2011). TMTT – (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, MVA - mevalonate, MEP - methylerythritol phosphate, ER - endoplasmic reticulum, P450 - cytochrome P450 monooxygenase, GES - geranyllinalool synthase, IPP - isopentenyl pyrophosphate, DMAPP - dimethylallyl pyrophosphate, GPP - geranyl pyrophosphate, FPP - farnesyl pyrophosphate, GGPP - geranylgeranyl pyrophosphate

Because SQT emissions from tomato plants are low and hardly detectable, most research of tomato constitutive emissions focuses on MT. MT are mainly diffusing out of the trichomes which act as storage-pools for these MT. Such pool emissions are predominantly temperature dependent but the amount of released MT is increased by trichome damage (mechanical damage by abiotic or biotic stresses). The release of constitutive MT emissions from an unstressed tomato is controlled by physicochemical properties involved in the diffusion process. One of the quantities determining such pool emissions is the concentration difference between the plant organs storing the MT and the surrounding air. As an approximation for the concentration difference, the vapour pressure difference between storing organ and atmosphere can be used. Typical vapour pressures of MT at physiologically relevant temperatures

are around $2 \cdot 10^{-2}$ bar (e.g. Tingey *et al.,* 1980) and in the air around $1 \cdot 10^{-10}$ bar *i.e.* negligible compared to the former. The other parameter determining the emission rates is the diffusive resistance between storing organs and air. In combination both, the diffusive resistance as well as vapour pressure difference determine the emission rates (Grote *et al.,* 2013; Tingey *et al.,* 1981).

1.1.2 Tomato induced BVOC emissions – biosynthesis, release and ecology

Besides constitutive emissions, in special situations, tomato plants also exhibit induced emissions. Induced emissions are generally triggered by stress, which may cause an increase in the emission rates by several orders of magnitude (Turlings *et al.,* 2004). These emissions reflect the activation of a large number of genes and activities in biosynthetic pathways as the plants' response to stress (Niinemets *et al.,* 2013). In tomato plants, the respective BVOC are released shortly after their synthesis (Farag and Paré, 2002). Induced volatiles are responsible for plant-insect and plant-plant interactions and their composition is dependent on the type of insect inducing the stress reaction (De Moraes *et al.,* 1998).

Well known induced emissions in tomato plants are: (*E,E*)-4,8,12-trimethyl trideca-1,3,7,11-tetraene (TMTT), (*E*)-β-ocimene, green leaf volatiles (GLV), methyl salicylate (MeSA), and SQT such as α-copaene.

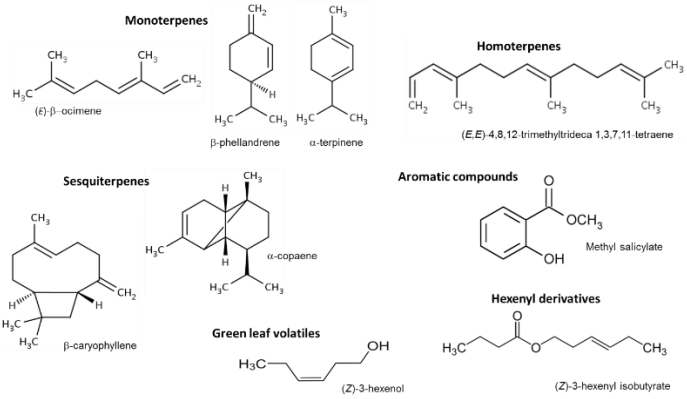


Figure 2 - Molecular structure of tomato induced emissions ((*E*)-β-ocimene, (*E,E*)-4,8,12-trimethyltrideca 1,3,7,11-tetraene, α-copaene, (*Z*)-3-hexenol, methyl salicylate, (*Z*)-3-hexenyl isobutyrate) and constitutive emissions (β-phellandrene, β-caryophyllene) with the highest emission rates.

TMTT is an acyclic homoterpene (Figure 2), formed by degradation of the 20-carbon precursor geranylgeranyl pyrophosphate (GGPP) in two enzymatic steps: formation of (*E,E*) – geranyllinalool followed by its oxidative degradation (Boland and Gabler, 1989; Tholl *et al.,* 2011). The first reaction is catalysed by terpene synthase, and the second by cytochrome P450 monooxygenase. The GGPP molecule is formed within the MEP pathway in plastids by condensation of IPP and DMAPP (for a review see Tholl *et al.,* 2011). In plastids, this molecule is a precursor for synthesis of other compounds such as diterpenes, carotenoids and gibberellins. Before GGPP can be used for TMTT synthesis, it needs to be carried out of plastids, first to the cytosol and then to the endoplasmic reticulum (ER), where the final two enzymatic steps elapse. The cytosol step is the crucial step for TMTT formation and it is characterized by the conversion of GGPP to (*E,E*) - geranyllinalool by (*E,E*) - geranyllinalool synthetase (Figure 1). In tomato plants, the regulation of TMTT synthesis in multiple steps has been found, especially for GGPP and its precursors (Kant *et al.,* 2004; Ament *et al.,* 2006). Furthermore, it has been demonstrated that synthesis of TMTT in tomato plants depends on both jasmonic (JA) and salicylic acid (SA) (Ament *et al.,* 2006), and it can attract natural enemies of herbivores during a parasite infestation (Kant *et al.,* 2004).

Another induced terpenoid is (*E*)-β-ocimene (Dicke *et al.,* 1990) (Figure 2). It is an acyclic MT emitted from plants after herbivore feeding and ozone exposure (Vuorinen *et al.,* 2004). *(E)*-β-ocimene is synthesized in the same way like other MT - via MEP pathway in plastids through condensation of IPP and DMAPP. In tomato, the proposed key step in *(E)*-β-ocimene synthesis is the formation of an intermediate linalyl cation which is then transformed into either (*E*)-β-ocimene or myrcene by mycerine/*(E)*-β-ocimene synthase (Bohlmann *et al.,* 2000). *(E)*-β-ocimene emissions are very common within plants such as cucumber, apple, lima bean, corn, potato, tobacco, and cotton, especially upon herbivore damage (Paré and Tumlinson, 1999; Dicke *et al.,* 1990; Turlings *et al.,* 1990; Röse *et al.,* 1996; Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002). (*E*)-β-ocimene emissions can be induced by JA or its derivatives (Horiuchi *et al.,* 2001; Birkett *et al.,* 2000), but not by simple mechanical injury (Arimura *et al.,* 2004). This indicates that this compound is not stored but *de-novo* synthesized. Its releases have also been reported from undamaged and insect damaged plants (Navia-Giné *et al.,* 2009; Degenhardt *et al.,* 2010) what makes (*E*)-β-ocimene a plant-plant signal molecule that influences JA pathway (Arimura *et al.,* 2004; 2002; Cascone *et al.,* 2014). Recently, it has been proven that (*E*)-β-ocimene is involved in attracting natural enemies (Zhang *et al.,* 2009; Arimura *et al.,* 2002), as well as pollinating insects (Pichersky and Gershenzon, 2002).

Upon pathogen infestation, tomato plants emit the SQT α-copaene (Figure 2) (Thelen *et al.,* 2005). α-copaene most likely plays a role in plant defence against pathogens, however more specific investigation of its role in plant communication so far have not been reported.

GLV is a group of induced BVOC. GLV emissions are detectable within minutes after membrane damage (Loreto *et al.,* 2006). The origin of GLV emissions are fatty acids (linoleic acid = 18:2 and linolenic acid = 18:3) that are set free as a result of membrane damage and their peroxidation by lipoxygenase (LOX) (Figure 3). Lipoxygenase further produces 9- or 13-hydroperoxylinoleic and -linolenic acid or a mixture of both. From the 13-hydroperoxylinole(n)ic acid, the products (*Z*)-3-hexenal (C_6-compound) and 12-oxo-(*Z*)-9-dodecanoic acid (C_{12}-compound) are formed by hydroperoxide lyase. Multiple LOXs and fatty acid hydroperoxide lyases, allow plants to synthesize GLV in several levels with additional modifications at each step for biosynthesis of alcohols and esters (Croft *et al.,* 1990; Heiden *et al.,* 2003). For example, from (Z)-3-hexenal compounds such as (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-3-hexenol or *(E)*-2-hexenal are formed while 9-hydroperoxidases produce nonenal, nonenol, nonadienal and nonadienol (Heiden *et al.,* 2003). In tomato plants, the strongest GLV emission is that of (*Z*)-3-hexenol (Farag and Paré,

2002) and its synthesis is presented in Figure 3. Reports show that JA treatment activates lipoxygenase (Blee, 2002) but GLV synthesis requires the presence of available free fatty acids (Figure 3).

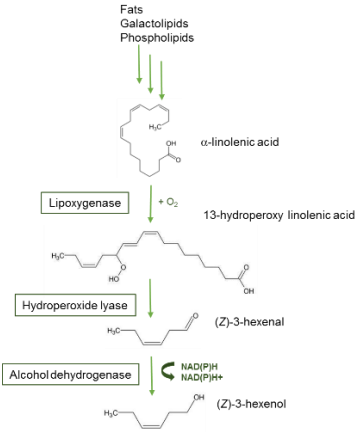


Figure 3 - Simplified overview of (*Z*)-3-hexenol biosynthesis.

GLV play a role in plants reactions to biotic stress and communication with other organisms (Arimura *et al.,* 2001; Farag and Paré, 2002; Dicke and Baldwin, 2010). GLV are toxic for herbivore insects (Arimura *et al.,* 2004) and for the plant itself. For example, in a plant cell (*E*)-2-hexenal is highly reactive with nucleophilic atoms, which are common in cellular proteins (Fall *et al.,* 1999; Farmer *et al.,* 2007). Plant fumigation with high concentrations of GLV might lead to toxic effects associated with necrosis development (Matsui *et al.,* 2012). In order to avoid their highly toxic effect, plants can transform GLV into less toxic compounds (Matsui *et al.,* 2012; Fujita and Hossain*,* 2003; Yan and Wang, 2006; Scala *et al.,* 2013).

MeSA is a volatile ester of the plant hormone SA (Figure 2). SA is generated downstream of the shikimate pathway either from benzoic acid, or from isochorismate (Lee *et al*., 1995; Wildermuth, 2001). MeSA is created by transfer of a methyl group, from the donor molecule *S*-adenosine-methionine to the carboxyl group of SA. Upon pathogen infection, many plants synthesize and accumulate SA in high levels (Yalpani *et al.,* 1991) and this process is involved in systemic acquired resistance (SAR). Once SA is transformed into MeSA, it can be used as an airborne signal involved in inducing disease resistance in

distant parts of the same plant or in neighbouring plants (Shulaev *et al.,* 1997). However, SAR is not induced by MeSA itself, but by SA. MeSA is taken up by distant plant parts and then converted back to SA (Kumar and Klessig, 2008). Except plant-plant communication, in tomato plants MeSA emissions can also attract natural enemies of herbivores (Dicke *et al.,* 1990). Furthermore, MeSA plays a role in defence against pathogens and it has been found to be toxic for microorganisms (Oloyede, 2011).

In general, induced emissions are *de-novo* emissions, i.e. the respective molecule is released into the atmosphere shortly after its synthesis. Several factors may influence their synthesis and emission rates. The factors with the strongest influence on *de-novo* BVOC emission rates are light, temperature (Niinemets *et al.,* 2010b; Hu *et al.,* 2013; Kesselmeier and Staudt, 1999) and the intensity of inducing stress factor (Niinemets, 2013). Furthermore, recent reports show that other factors might also have an impact on plant induced BVOC emissions. Such factors are atmospheric CO_2 concentrations (Raisanen *et al.,* 2008; Velikova *et al.,* 2009; Sun *et al.,* 2012), or leaf and plant age (Mayrhofer *et al.,* 2005; Guenther *et al.,* 2006; Sun *et al.,* 2009; Niinemets *et al.,* 2010a; Sun *et al.,* 2012; Shiojiri and Karban, 2006).

1.1.3 BVOC emissions and induced plant defence

Plants are exposed to different types of stress including pathogen or herbivore attacks. In order to fight such biotic stresses plants have developed a set of different defence mechanisms.

Plant defence is a set of constitutive and induced strategies. In tomato plants, constitutive defence also includes trichomes and constitutive BVOC (Kang *et al.,* 2010) emissions.

Once this physical barrier (cuticula) has been crossed, plants can activate another type of defence termed inducible defence. Inducible defence involves many hormones with specific downstream responses. Plants induced BVOC emissions are a result of activation of such a signalling pathway. SA, JA and ethylene are intensively investigated plant hormones (Arimura *et al.,* 2005; Derksen *et al.,* 2013; Ton *et al.,* 2002; Cui *et al.,* 2012). Other plant hormones such as abscisic acid (Cao *et al.,* 2011; Nakashita *et al.,* 2003) and auxins (Navarro *et al.,* 2008) also play a role in steering plant defence responses. Salicylic response is associated with development of hypersensitive response and programmed cell death. In general, this type of defence strategy is

associated with plant defence against biotrophs (pathogens that feed on living cells) (Smith *et al.,* 2009). On the other hand, plants defence against necrotrophs (pathogens that live and feed on the dead cells) is associated with activation of the JA signalling pathway (El Oirdi *et al.,* 2011). Similar general processes have been observed in plants reaction to herbivores. Many sucking insects activate the SA pathway, while plants reaction to chewing insects is usually associated with JA pathway (Stout *et al.,* 2006). However, plant defence is established by a crosstalk between SA and JA pathways that can be either antagonistic or synergistic (Robert-Seilaniantz *et al.,* 2011; Mur *et al.,* 2006; Smith *et al.,* 2009) and it allows a plant to fine-tune its defence against various biotic stressors.

Application of MeJA or ozone has a similar impact on plants as pathogens and parasites. MeJA and ozone exposures also can activate signalling pathways in plants. MeJA is a JA derivative, used by plants as an airborne signal molecule for activation JA pathway in distant plant parts (Kawano *et al.,* 2013; Repka *et al.,* 2001; Cheong and Choi, 2003). The JA pathway is associated with the release of secondary metabolites that can interfere with herbivore feeding and digestion (Chen *et al.,* 2005) or induce emissions of BVOC (Semiz *et al.,* 2012) that can repel herbivores (De Moraes *et al.,* 2001) and attract their natural enemies (Turlings *et al.,* 1990).

Ozone exposure is known to mimic biotic stress in plants, leading to activation of hypersensitive response or systemic acquired resistance (Sandermann *et al.,* 1998). This kind of plant reaction to stress is mediated by activation of JA and ethylene (van Wees *et al.,* 2008) and/or SA pathway (Sticher *et al.,* 1997). Exposing plants to ozone also leads to the formation of reactive oxygen species (ROS) (Sandermann, 1996; Schraudner *et al.,* 1997). Plants take up ozone through stomata and decompose it in the apoplast. Exposures of high ozone concentrations can cause oxidative bursts (Pell *et al.,* 1997) which might trigger a signal for hypersensitive response (Sandermann *et al.,* 1998). Upon ozone exposure, plants can emit different compounds originating from the SA signalling pathway, from which the most common is MeSA (Heiden *et al.,* 1999). Typical visual symptoms of ozone damage is development of necrotic spots that causes membrane damage (Heiden *et al.*,1999) and synthesis of JA – derivatives in the octadecanoid pathway (Arimura *et al.,* 2005).

1.2 Problem description

Tomato is a second most consumed vegetable in the world and one of the economically most important crop species after maize, rice, wheat, potatoes, soybeans and cassava (Bergougnoux, 2014). European tomatoes are produced in field (mostly for preserves) and in greenhouses (off-season vegetables, mostly for fresh market). Both production systems are facing numerous problems that will have severe consequences on future tomato production.

European largest field tomato production is in regions with semiarid and Mediterranean climate (Gould, 1991). These regions are already starting to experience problems in tomato production caused by severe droughts due to climate change (Peñuelas *et al.*, 2009; IPCC, 2007). Furthermore, the future prognosis indicates even more extreme conditions due to climate change. Such drought periods will severely limit, if not completely prevent, field tomato production in Mediterranean areas (IPCC, 2007).

In modern greenhouses, factors such as temperature, light, air humidity, CO_2 concentration, water supply and nutrients can be adjusted to meet demands of specific growing crops. Greenhouse technology does not only allow growing crops in areas where outdoor field production is limited due to unfavourable climate, but also has a potential to extend the growing season for crop production and higher yields. Therefore, it is possible that future European tomato production will be fulfilled mostly in greenhouses rather than in the field. This kind of technology has its disadvantages such as high resource, finance and labour input, what sometimes makes even greenhouses with organic production unsustainable (EGTOP, 2013). Guided by global climate change and environment protection, new European political decisions are challenging the greenhouse production. One of these challenges is the "Water Framework Directive" that aims to raise water quality in Europe including the regulation of the amount of nutrients and pesticides released from greenhouses into the environment. This directive will force numerous greenhouse growers to limit use of conventional methods of controlling biotic stress in greenhouses and turn to alternative methods for keeping their yield at an optimal level.

1.2.1 Objective

The focus of this thesis is the investigation of tomato BVOC emissions in order to assess whether or not the knowledge on such emissions can give advantages for future tomato growing industry. Aim of this work is to provide a broad tomato BVOC emission study that can give an answer to following questions:

1. What are the impacts of drought on constitutive and induced BVOC emissions from tomato? Can BVOC emissions be used as an indicator for the onset of drought?

2. What are the impacts of biotic stress on tomato BVOC emissions? Can BVOC emissions be used for early detection of biotic stress? If so, what are the target compounds for early stress detection in greenhouses?

2 GENERAL METHODS

In this chapter, the general methods and the experimental setup are described, which are common for all experiments. Additional methods or special experimental setups that are unique for the respective experiments will be described in the respective chapters.

2.1 Plant material

All tomato plants *Solanum lycopersicum* cv. Moneymaker were grown in a growth room at 20 °C and under artificial light with intensities between 300 and 600 µmol· m^{-2}·s^{-1} and a diurnal rhythm of 14 h light and 10 h darkness. Plants were sown directly into 400 ml pots containing 570 g of substrate (Einheitserde Typ VM). They were watered daily with a 2 % nutrient solution (Kristalon rot Calcium 11+11+24, Yara Dülmen, Germany). Gas measurements started when the plants were about four weeks old if not mentioned otherwise.

2.2 Experimental system setup

Experiments were carried out in the Jülich Plant Atmosphere Chamber (JPAC) facility at Forschungszentrum Jülich, Germany as described in detail by Heiden *et al.* (2003), Schimang *et al.* (2006), and Wu *et al*. (2015) (Figure 4). Four newly constructed borosilicate glass chambers (volume 13L) each paired with an LED lamp (LED Light Source SL3500-W-J, Photon Systems Instruments, Drásov, Czech Republic) were located in a climate-controlled housing (stability ± 0.5°C). At typical mid-canopy heights, photosynthetic photon flux density (PPFD) was adjusted to 400 µmol·m^{-2}·s^{-1} and held constant during periods of illumination (14 hours). Depending on the size of the plants, the airflow through the chamber was adjusted to 7-10 l·min^{-1} using digital mass flow controllers (Bronkhorst High-Tech B.V., Ruurlo, The Netherlands).

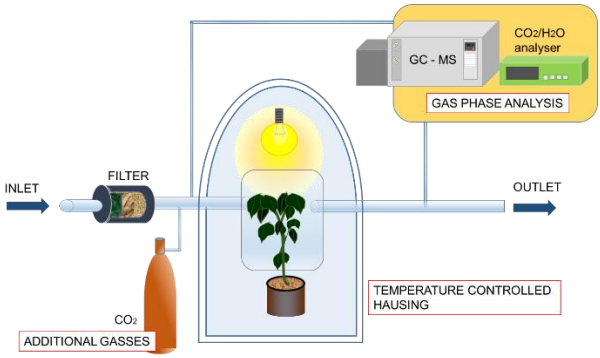


Figure 4 - A simplified schematic presentation of JPAC system setup.

The air entering the chambers was purified by an adsorption dryer (KEA 70; Zander Aufbereitungstechnik GmbH & Co. KG, Essen, Germany) and by a palladium-catalyst running at 450 °C. Mixing ratios of water vapour and CO_2 were reduced to 0.3 % and ~ 70 ppm, respectively and mixing ratios of VOC were diminished to below the detection limit of the GC-MS instruments (less than 1 ppt).

Before the air was led into the chamber, CO_2 was added from a pressurized cylinder. With plants in the chamber, the CO_2 concentrations in the chamber were kept constant in the range of 350 to 400 ppm, depending on light intensity and on the leaf area of the investigated individual plant. Due to transpiration of the investigated plants, the dew point in the chamber was around 15 -17 °C during light periods (equivalent to a relative humidity of 73-83 % at the chamber temperature of 20 °C). Differences in CO_2 and water vapour concentrations between inlet and outlet of the chambers were measured by IR absorption (Li-Cor CO_2/H_2O analyser, Lincoln, Nebraska, USA). Absolute water vapour concentrations were measured by a dew point mirror (MTS-MK1, Walz, Effeltrich, Germany). BVOC were measured at the outlet air of the plant chamber by a GC-MS system optimized for C_5 to C_{20} BVOC including MT and SQT as well as GLV or MeSA.

Except for whitefly (WF) tests, BVOC analysis was conducted by JPAC GC-MS systems as described in detail by Heiden *et al.* (2003). This system was based on HP 5890 Series II gas chromatography with a quadruple mass selective detector, HP-MSD 5972A. The GC system used thermal adsorption/desorption for pre-concentration (Gerstel online TDS G) connected

to a cooled injection system (Gerstel; KAS 3). Samples were cryofocused before the injection onto the column (BPX-5 column, SGE, 50 m · 0.22 mm · 1µm). This GC-MS system and its calibration are described in detail by Heiden *et al*. (2003).

Gas analysis for WF tests was conducted by using another GC-MS system set in facilities of Plant Research International, Wageningen University and Research Centre. In this second GC-MS system, volatiles were analysed with a Thermo Trace GC connected to a DSQ mass spectrometer (Thermo Fisher Scientific, USA). Volatiles were collected at 4°C on an electronically cooled sorbent trap (Unity, Markes, Llantrisant, UK). These were then transferred in split mode (1:5) to the analytical column (ZB-5Msi, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Phenomenex, USA) by rapid heating of the cold trap to 250°C for 6 min. The gas sampling for plants tested in the WF experiment, including controls, lasted 24 hours. This GC-MS system did not have a calibration system.

Each plant was introduced into the outer climate-controlled housing 24 hours before starting the BVOC measurements in order to allow plant adaptation to the light and temperature settings. After that, each individual plant was placed inside the measuring chamber. For most plants, it took another day to adapt and stabilize its transpiration rate. In each chamber, a Teflon sheet was used to separate the gas phase around the shoot from roots and substrate to prevent contamination of the chamber air by emissions from substrate and roots. The plant stem was positioned through a hole in the middle of the Teflon sheet and sealed airtight with the elastic material Optosil P (Heraeus Kulzer GmbH, Hanau, Germany).

Flux densities Φ(X) of each monitored compound (X) were calculated as described in Wu *et al.* (2015). To calculate Φ(X), the differences of mixing ratios between outlet and inlet air of the measuring chamber, the air flow through the chamber, F_{air} [mol·s^{-1}] and leaf area A_{leaf} [m^2] were used as the base:

$$\Phi(X) = \frac{F_{air} \cdot ([X] - [X]_i)}{A_{leaf}} \quad (1)$$

In equation (1), [X] is the mixing ratio of compound (X) at chamber outlet and $[X]_i$ is the mixing ratio of the compound at chamber inlet. Transpiration rates and net assimilation rates were calculated by using the same formula.

According to previous tests (e.g. Schuh *et al.,* 1997; Heiden *et al.,* 2003; Schimang *et al.,* 2006), wall losses and chemical reactions were negligible. $[X]_i$ was set to zero for the BVOC.

All uncertainties of data are presented by using standard errors. Data were compared by using two-tailed T-test.

3 INDUCTION OF BVOC EMISSIONS BY METHYL JASMONATE AND OZONE EXPOSURE

3.1 Introduction

Biotic stresses are difficult to define and to control, even under laboratory conditions. Plant and parasite/pathogen performance is affected by a variety of different factors and innate biological variability (e.g. Elad *et al.,* 1995) causing high variations between data and at times unclear results. Furthermore, pathogens and parasites can manipulate plant-signalling pathways resulting in conflicting results (El Oirdi *et al.,* 2011; Giordanengo *et al.,* 2010). To avoid variations in impact that living organisms might have on plant, studies investigating plant defence reactions rely mostly on stressors that can easily be applied and controlled. Such studies often include exposing plants to MeJA or ozone as a tool that can activate plants signalling pathways and mimic biotic stress (Pauwels *et al.,* 2009; Gómez *et al.,* 2010; van Dam *et al*., 2001; Vuorinen *et al.,* 2004; Sandermann, 1996; Heiden *et al.,* 1999) and such exposures can be applied in a reproducible manner.

MeJA exposure activates the JA pathway in plants (Repka *et al.,* 2001; Cheong and Choi, 2003). In a similar way, plants activate both the JA and the SA pathways upon ozone exposure (Sandermann *et al.*, 1998).

In this study, tomato plants were exposed to ozone and MeJA in order to find the best method for further studies on induced BVOC emissions. Furthermore, BVOC emissions from MeJA treated plants were compared to BVOC emissions induced by ozone. The purpose of these experiments was to answer the following research questions:

1. Which BVOC emissions are induced in tomato plants after MeJA exposure?

2. Which BVOC emissions are induced in tomato plants after ozone exposure?

3.2 Specific materials and methods

3.2.1 Ozone exposure

Each plant was first introduced into the measuring chamber and after constitutive BVOC emissions had stabilized, the plant was exposed to ozone. O_3 was produced by photolysis of oxygen using a UV light source (Pen-Ray, UVP, Inc., Upland, CA, USA, □=189 nm). Air entering the measuring chamber was first mixed with O_3. Plants were exposed to O_3 for about one hour. In order to identify all induced compounds emitted after O_3 exposure, seven plants were exposed to different ozone concentrations ranging from 230 to 1750 ppb. For investigating temporal shapes of BVOC emissions from plants under severe but comparable ozone stress, additional six plants were exposed to maximum ozone concentrations around 1500 ppb for one hour. After stopping an ozone exposure, it took about 10 minutes (depending on the gas flow) for ozone concentrations to drop to below 40 ppb allowing beginning of BVOC measurements by GC-MS (Table 1).

Table 1 - Overview of ozone and methyl jasmonate experiments

Treatments	Time of exposure before gas measurement [h]	Number of plants
Ozone 230-1750 ppb	**1**	**7**
Ozone 1500 ppb	**1**	**6**
Methyl jasmonate 0,05 ml	**0**	**6**
Control - no treatment	**-**	**6**

3.2.2 MeJA exposure

Six plants were exposed to MeJA. After constitutive BVOC emissions had stabilized, MeJA treatment was applied. A filter paper soaked with 0,05 ml MeJA solution (Sigma-Aldrich, St. Louis, USA; purity > 95 %) was placed on the bottom of the chamber without any contact to the plant. The MeJA soaked filter paper was not removed until the end of the experiment. BVOC measurement lasted for 24 hours under constant light.

3.3 Results

3.3.1 Control plants

In all control plants, dominant emissions were constitutive MT and the homoterpene (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). Detected constitutive MT emissions were limonene, α-pinene, α-terpinene, γ-terpinene, β-pinene, p-cymene and β-phellandrene as the strongest MT emission. All detected MT emissions were almost perfectly correlated with each other (R^2 >0.9, Figure 5) indicating that all these MT emissions were based on the same mechanism. Therefore, one of these emissions could be used as proxy to demonstrate the behaviour of all other MT emissions (compare to Wu *et al.,* 2015). As representative of constitutive MT emissions, emission rates for α-terpinene was chosen, since it was one of the strongest emissions and in chromatograms its peak did not overlap with that of any other MT. In all control plants, MT emissions showed minor fluctuations but with no systematic trend during the measurement time.

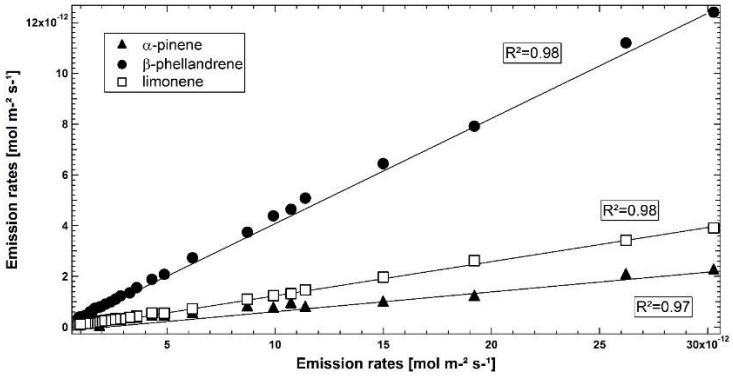


Figure 5 - Plot emissions of the monoterpenes α-pinene, β-phellandrene and limonene in dependence of α-terpienene emissions after plant touching. All monoterpenes are also emitted constitutively. To increase the dynamic range of emissions the plant was touched causing trichome damage.

TMTT emissions in control plants were below the detection limit of the analytical device for several hours after introducing the plant into the measuring chamber and thereafter they slowly but constantly increased.

SQT emissions, such as β-caryophyllene, were very low and close to detection limit of the analytical device (~ 1 ppt). Except constitutive MT, TMTT and the minor amounts of β-caryophyllene, no other compounds were detected in the emissions from control plants.

3.3.2 Ozone exposed plants

In experiments with different ozone concentrations, most plants developed typical visual symptoms of ozone damage such as necrotic spots. Only in plants exposed to the lowest ozone concentrations (230 and 400 ppb), no visual symptoms were observed. The most severe leaf damage was observed in plants exposed to the highest ozone concentrations (1500 ppb or higher).

The number of detectable emissions increased with increasing ozone concentrations (Table 2). Similar temporal shapes were found for the emissions

of all detected compounds and in all tested plants: emissions reached their maximums directly or few hours after the ozone exposure and thereafter decreased with time (Figure 6 and Figure 7).

Table 2 - Overview of detected compounds and their highest emission rates in tomato plants exposed to different ozone concentrations. GLV - green leaf volatiles, cMT – constitutive monoterpenes, iMT - induced monoterpenes, MeSA - methyl salicylate, TMTT - (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, HexD - hexenyl derivatives, - - emission rates below $1 \cdot 10^{-16}$ $mol \cdot m^{-2} \cdot s^{-1}$

Ozone	ppb	230	400	940	1250	1500	1550	1750
	Φ mol· $m^{-2} \cdot s^{-1}$	$4.7 \cdot 10^{-10}$	$8.3 \cdot 10^{-10}$	$1.4 \cdot 10^{-9}$	$1.5 \cdot 10^{-9}$	$1.7 \cdot 10^{-9}$	$2.7 \cdot 10^{-9}$	$4.7 \cdot 10^{-8}$
Detected emissions $mol \cdot m^{-2} \cdot s^{-1}$	GLV (*Z*)-3-hexenol	-	-	$7.0 \cdot 10^{-14}$	$7.5 \cdot 10^{-14}$	$1.6 \cdot 10^{-13}$	$7.9 \cdot 10^{-12}$	$6.2 \cdot 10^{-11}$
	cMT α-terpinene	$1.6 \cdot 10^{-13}$	$3.0 \cdot 10^{-13}$	$8.4 \cdot 10^{-13}$	$1.1 \cdot 10^{-12}$	$5.0 \cdot 10^{-12}$	$5.3 \cdot 10^{-12}$	$6.0 \cdot 10^{-12}$
	iMT (*E*)-β-ocimene	$9.4 \cdot 10^{-15}$	$2.0 \cdot 10^{-14}$	$1.1 \cdot 10^{-14}$	$2.7 \cdot 10^{-14}$	$5.5 \cdot 10^{-14}$	$1.8 \cdot 10^{-14}$	$1.5 \cdot 10^{-14}$
	MeSA	-	-	$3.3 \cdot 10^{-14}$	$3.8 \cdot 10^{-14}$	$1.1 \cdot 10^{-13}$	$2.9 \cdot 10^{-13}$	$3.8 \cdot 10^{-13}$
	TMTT	$6.4 \cdot 10^{-14}$	$8.3 \cdot 10^{-14}$	$5.5 \cdot 10^{-14}$	$6.9 \cdot 10^{-14}$	$1.6 \cdot 10^{-14}$	$2.0 \cdot 10^{-13}$	$5.7 \cdot 10^{-14}$
	HexD (*Z*)-3-hexenyl isobutyrate	-	-	-	-	$5.2 \cdot 10^{-13}$	$1.2 \cdot 10^{-12}$	$7.6 \cdot 10^{-12}$

In plants without any visual symptoms of ozone damage, GLV emissions were not detected. In all other plants, several different GLV were detected with (*Z*)-3-hexenol as a dominant one. All detected GLV were strongly correlated with each other (R^2 >0.9), therefore (*Z*)-3-hexenol was chosen as representative of GLV emissions.

A new group of gases was detected only from plants treated with the highest ozone concentrations (1500 ppb and higher). These gases were termed hexenyl derivatives (HexD) and they included *(Z)*-3-hexenyl propanoate, *(Z)*-3-hexenyl butyrate, *(Z)*-3-hexenyl isobutyrate, *(Z)*-3-hexenyl valerate and *(Z)*-3-hexenyl isovalerate. Since all detected HexD emissions were correlated to each other (R^2 >0.9), (*Z*)-3-hexenyl isobutyrate was used as a HexD representative. In plants, where both GLV and HexD were detected, these two emission groups were not correlated (Table 2).

All six plants exposed to 1500 ppb of ozone emitted identical compounds. Emissions included those of GLV, HexD, MeSA, TMTT and (*E*)-β-ocimene. The temporal shapes of emissions were very similar between plants. During ozone exposure, transpiration dropped by about 50 % and for next several hours it showed no or only minimum recovery.

In all plants, regardless of ozone concentrations, emissions of MT from storage pools were strongest right after the ozone exposure, followed by a slow decrease to the level of control plants (Figure 6). In plants that emitted GLV, behaviour of GLV was similar to behaviour of MT emissions – emissions were strongest right after ozone exposure, followed by their decrease to below the detection limit (Figure 6). Detected maxima of MT and GLV emission rates were the higher as the higher were the concentrations of applied ozone (Table 2).

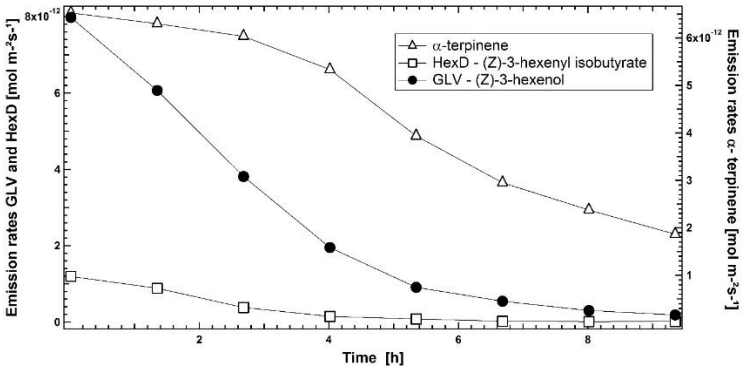


Figure 6 - Example of a temporal shape of α-terpinene, (*Z*)-3-hexenyl isobutyrate and (*Z*)-3-hexenol emissions from severely ozone stressed plant (1550 ppb)

(E)-β-ocimene was detected in all ozone-exposed plants. After ozone exposure, *(E)*-β-ocimene emissions were increasing for 2-3 more hours and thereafter they started to decrease (Figure 7).

In plants exposed to lower ozone concentrations (230 and 400 ppb), MeSA emissions were not detected. In all other ozone treated plants, MeSA emissions were above the detection limit and showed similar temporal shapes between plants: after ozone exposure, MeSA emissions were slightly increasing for next 1-2 hours and decreased thereafter (Figure 7).

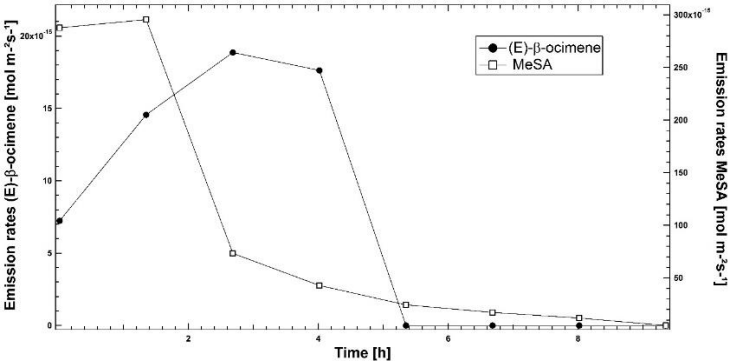


Figure 7 - Example of (*E*)-β-ocimene and MeSA emissions from tomato exposed to ozone (1550 ppb); MeSA – methyl salicylate

TMTT emissions from ozone stressed and control plants were very similar until ozone application. They increased steadily with time. After ozone exposures, the TMTT emissions differed from those observed for control plants. In ozone treated plants, TMTT increased for few hours right after ozone exposure and thereafter they slowly decreased. This behaviour was observed for all ozone treated plants (data not shown).

3.3.3 MeJA exposed plants

In plants exposed to MeJA no obvious visual symptoms were observed within 24 hours after exposure. Two plants were left in the chamber for a longer period of time. In those plants, first visual symptoms (yellowing of the youngest leaves) occurred almost one week after MeJA exposure.

GLV and MeSA emissions were not detected in plants exposed to MeJA. There were also no obvious differences in TMTT emissions between MeJA exposed and control plants. Directly after introducing plant into the measuring chamber, TMTT emissions were below the detection limit for several hours. They started to increase before MeJA application. MT and TMTT emissions seemed unaffected by MeJA treatments.

The only obvious difference between control plants and MeJA treated plants was the presence of HexD, (*E*)-β-ocimene and α-copaene. (*E*)-β-ocimene was detected in average 14.8 ± 6.6 hours after introducing MeJA into the measuring chamber, while (*Z*)-3-hexenyl isobutyrate emissions were detected later, average 20 ± 10.6 hours after exposure. Both of these BVOC emissions increased with time. α-copaene was present in all MeJA exposed plants, but its emission rates were very low and not detectable in all chromatograms.

3.4 Discussion

Bursts of GLV emissions and increased release of MT stored in pools were not specific for ozone exposure. GLV emissions, after membrane damage, appear independent from the cause of membrane damage (Croft *et al.,* 1993; Heiden *et al.,* 2003). Previous reports show that GLV emission strengths are related to the severity of wounding (Fall *et al.,* 1999), to the formation of necrotic spots (Behnke *et al.,* 2009), and to ozone uptake rates (Beauchamp *et al.,* 2005). The relationship observed here between ozone concentrations and GLV emissions is consistent to reports in literature.

Jansen *et al.* (2009a) reported a correlation between development of necrotic spots and amount of emitted GLV in tomato plants. My findings are in agreement with Jansen *et al.* (2009a) showing that less tissue damage causes lower GLV and MT emissions, and vice versa.

Treating plants with MeJA neither induced GLV emissions nor affected the release of stored MT. Bursts of GLV emissions require actual membrane damage (Croft *et al.,* 1990; Heiden *et al.,* 2003) and in tomato, increased MT releases from pools require trichome breakage. I therefore conclude that MeJA exposures at the concentrations and time periods as used here, do not cause substantial membrane damage nor cause trichome damage.

Experiments with different ozone concentrations indicate the existence of a threshold level for the induction of high HexD emissions. In tests with high ozone concentrations (around 1500 ppb), HexD were emitted always together with GLV. Similar observations have been reported in tomato plans during herbivore feeding, when HexD were detected together with GLV and therefore often referred as gas emissions related to GLV (Raghava *et al.,* 2010; Degenhardt *et al.,* 2010). In ozone tests, GLV and HexD emissions were not correlated to each other, showing that the emissions of HexD were independent of GLV emissions. Furthermore, in MeJA exposures, HexD emissions were detected hours after the MeJA treatment, but without any observable GLV emissions. Obviously, MeJA exposure was sufficient for inducing HexD emissions but not for inducing GLV emissions. However, HexD plus GLV were emitted right away after ozone exposure. As in the case of ozone, it is possible that exceeding a threshold level was also required to induce HexD emissions during MeJA exposure. Constant presence of MeJA in the measuring chamber might result in a prolonged mild stress that might slowly reach such threshold for inducing HexD emission (Niinemets *et al.,* 2010a). Therefore, late HexD emissions may not necessarily be the consequence of a late reaction to MeJA, but rather plants reaction to prolonged mild stress. MeJA itself seems to be enough to trigger HexD emission, but ozone might influence the speed of plant response by reaching stress threshold for HexD emissions sooner. It is possible that HexD synthesis is regulated on multiple levels by different signalling pathways what might have caused the differences of the results between ozone and MeJA treated plants.

Once induced by MeJA exposure, (*E*)-β-ocimene and HexD emissions were both steadily increasing. This results show that (*E*)-β-ocimene emissions are most likely induced by MeJA. However, just like in the case of HexD, in combination with other pathways or by supporting JA pathway trough GLV, (*E*)-β-ocimene emissions seem to be triggered earlier. At this point, reasons for differences in timing of (*E*)-β-ocimene and HexD induction between ozone and MeJA treatments are unknown. These experiments show that MeJA induced compounds such as HexD and (*E*)-β-ocimene were still emitted in ozone treated plants when both JA and SA pathways were active.

Increase in TMTT emissions from control plants and MeJA treated plants was very similar. However, impact of ozone exposure on TMTT emissions could not be studied due to gas measurement interruption during ozone exposure. None of the tested plants (MeJA exposed, ozone exposed or controls) had TMTT emissions above the detection limit at the moment when they were introduced into the measuring chamber. In all tested plants TMTT emissions slowly increased after the plants were introduced in the chamber and before plant treatment. Therefore, the first induction of TMTT emissions cannot be associated with application of MeJA or ozone. I assume that TMTT emissions are a result of unidentified stress inside the measuring chamber. It is possible that such a stress can be caused by some less favourable growing conditions inside the measuring chamber. For example, due to measuring chamber design, plant leaves that are closest to the gas inlet of the measuring chamber may suffer the direct exposure of a very dry airflow. Whether this kind of mild stress in a longer period can induce TMTT emissions, still needs further investigation. Furthermore, reports on TMTT are in agreement with here presented findings, showing that TMTT emissions in tomato plants are stress induced (e.g. Farag and Paré, 2002; Thaler *et al.,* 1996; Ament *et al.,* 2006).

As ozone exposures activate the SA pathway in plants (Sandermann, 1996), it is expectable that ozone stressed plants emit MeSA, since MeSA originates from SA signalling pathway (Lee *et al.,* 1995). MeJA treatment activates JA pathway (Chen *et al.,* 2006) but not the SA pathway, what explains lack of MeSA emissions in MeJA treated plants.

α-copaene emissions were detected only during MeJA exposure but not after ozone exposures. However, ozone exposure in plants triggers both SA and JA pathway (Sandermann, 1996). Hence, α-copaene emissions might be expected also after ozone exposures. Not detecting α-copaene emissions after ozone exposure might be explainable by stomata closure during ozone exposures or by antagonistic cross talks of the SA and the JA pathway.

The here presented results confirm that emissions of α-copaene, HexD, (*E*)-β-ocimene, TMTT and MeSA are stress induced. These stress-induced emissions decrease within few hours after ozone exposure was stopped and transpiration rate was already reduced by 50 %. Decreasing transpiration is explainable by stomata closure (Thwe *et al.,* 2014) and leaf damage due to ozone exposure. The purpose of this study was to identify induced compounds after ozone exposure; I therefore did not put too much attention to the differences in timing between decrease of induced BVOC emissions and decrease of transpiration.

Further tests were made to exclude that tomato possesses storage pools for α-copaene, HexD, (*E*)-β-ocimene, TMTT and MeSA. Plants were subject to harsh handling causing membrane and trichome damage what resulted in bursts of GLV and release of MT emissions from storage pools. No emission pulses were found for α-copaene, HexD, (*E*)-β-ocimene, TMTT and MeSA indicating that tomato does not contain storage organs for these compounds.

3.5 Summary and conclusions

Exposing plants to ozone or to MeJA respectively caused different responses of tomato. While both stressors induced emissions of (*E*)-β-ocimene and HexD, ozone exposure additionally induced emissions of GLV and MeSA, and increased constitutive MT emissions. MeJA exposures additionally induced emissions of α-copaene.

These tests were made to control whether or not these stressors can be used to first induce the respective emissions and thereafter control the impacts of drought on the induced emissions. As result, MeJA exposure seemed suitable due to its induction of high and longer lasting BVOC emissions with no severe plant damage. Ozone exposures caused too much leaf damage to allow any reliable conclusions on the impact of drought. Therefore, ozone exposures were not suitable for inducing BVOC emissions for further drought studies.

On the other hand, it was aimed at testing if such induced emissions allow conclusions on the signalling pathways induced by the stressors. Ozone exposures caused emissions of GLV. As GLV are produced in the octadecanoid pathway that also leads to the formation of JA, the appearance of (*E*)-β-ocimene and those of HexD is not surprising. Ozone exposures also induced MeSA emissions and thus, induction of the SA pathway by ozone exposure is probable. Ozone exposure therefore induced both, the JA and the SA pathway, but a contribution of the SA pathway to the induction of (*E*)-β-ocimene and of HexD emissions cannot be ruled out.

MeJA exposures did not induce MeSA emissions from tomato suggesting that induction of the SA pathway by MeJA is of minor importance. However, it is unknown if (*E*)-β-ocimene and HexD emissions can be induced by any other pathway besides JA pathway. Therefore, at this point no definite conclusions can be drawn on the signalling pathways related to (*E*)-β-ocimene or HexD emissions.

For MeSA and GLV emissions, the situation is somewhat different. MeSA emissions originate from SA (Lee *et al.,* 1995; Wildermuth, 2001). The appearance of MeSA emissions strongly hints to an induction of the SA pathway. GLV are produced within the octadecanoid pathway (Croft *et al.,* 1990) and membrane damage is required to induce this pathway. The appearance of GLV emissions therefore is a hint to the induction of the octadecanoid pathway.

The finding that TMTT emissions were not changed by MeJA exposures indicates that JA pathway is not an efficient trigger for TMTT emissions. TMTT emissions from tomato must be induced by another metabolic pathway.

Another difference in the emission response of tomato to ozone and MeJA exposures, respectively, were the increased releases of MT stored in trichomes after ozone exposures. While MeJA exposures did not induce strong leaf damage and leaf wilting, ozone exposures did. A mechanical destruction of trichomes, as reason for the increased release of constitutive MT after ozone exposures, is therefore probable (compare also drought induced increases of constitutive MT emissions, Chapter 4).

4 EFFECT OF DROUGHT STRESS ON CONSTITUTIVE AND INDUCED BVOC EMISSIONS FROM TOMATO

4.1 Introduction

Most studies regarding impacts of drought deal with constitutive emissions like isoprene or MT. Impacts of drought on induced emissions are less studied. Gouinguene and Turlings (2002) reported higher total emissions when wounding plants that were grown at lower soil humidity. They also found changes of the emission patterns indicating different behaviour for different BVOC.

The research question in this chapter was to assess, how drought affects BVOC emissions in quality and quantity. The experiments focused on the dynamic behaviour of induced emissions. The purpose of this study was to test the hypothesis, that BVOC emissions can be used as indicators for the early drought stress.

4.2 Specific materials and methods

4.2.1 Drought application and monitoring

Table 3 shows an overview of the experiments made with respect to drought application. The experiments were conducted in the following manner: in order to provide identical starting conditions for all drought treatments, each plant was watered until 100 % water holding capacity (WHC), and then introduced into the measuring chamber. Thereafter, the plants were not watered any more. Control plants were watered daily in order to compensate for water loss by transpiration. Control plants held under permanent light were watered with 125 ml of water per day while control plants under diurnal light settings were watered with 80 ml of water per day.

Table 3 - Overview of experiments regarding impacts of drought on BVOC emissions from tomato; MeJA - methyl jasmonate; light settings: D/N - day and night, PL - permanent light; investigated BVOC: cMT - constitutive monoterpene emissions, iMT - induced monoterpene emissions, TMTT - (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, GLV - green leaf volatiles, HexD - hexenyl derivatives

Experiment		Light settings	Number of experiments	Adaptation time Hours before beginning of the experiment	Investigated BVOC
Without MeJA exposure	Drought	D/N	5	0	cMT, TMTT, GLV
		PL	7	24	cMT, TMTT, GLV
	Control	D/N	4	0	cMT, TMTT, GLV
		PL	4	24	cMT, TMTT, GLV
With MeJA exposure	Drought	D/N	4	0	cMT, iMT, TMTT, GLV, HexD
		PL	3	24	cMT, iMT, TMTT, GLV, HexD
	Control	D/N	5	0	cMT, iMT, TMTT, GLV, HexD
		PL	1	24	cMT, iMT, TMTT, GLV, HexD

Four series of measurements were conducted to determine the impact of drought on BVOC emissions. In two series, I tested the impact of drought on constitutive emissions and on TMTT emissions. In one of these series, the plants were exposed to a diurnal variation of light (14 h illumination, 10 h darkness) and in the other series the plants were exposed to permanent light.

The other two series of measurements were conducted with plants exposed to MeJA. In one series, light intensity had a diurnal variation (14 h illumination and 10 h darkness). In the other series, experiments were conducted under permanent light. MeJA exposure was used to elicit emissions of (*E*)-β-ocimene and HexD.

All drought and control experiments lasted between five and eight days.

In order to avoid losing important data, in experiments with day and night settings, adaptation time was included in the results. For all other experiments, results obtained during the adaptation period are not reported (Table 3).

The substrate's water holding capacity was determined in six experiments independent of BVOC measurements (not included in Table 3). Pots with 570 g of dry substrate and plants at the same age as in the other experiments were first soaked with water for 24 hours and then left to drain for another 24 hours for removal of water from the macropores. Pots with substrate and plants were sealed on top with plastic foil and kept dark in order to prevent any water loss due to evaporation or transpiration. After this period, pots with plants were weighed what led to an average total mass of 900 g (± 41g).

4.3 Results

4.3.1 Transpiration rates as a plant drought status indicator

In order to characterize the severity of drought, I tested if positioning the plant on the balance during gas measurements can be used for monitoring water loss from substrate. Investigating BVOC emissions from plants positioned on a balance caused problems when the plants were re-watered. Most likely, the plants stem was injured due to small movements of the balance inducing GLV emissions. Therefore, characterization of the severity of drought by substrate water content was not feasible. Instead, transpiration rates were used to monitor drought conditions in relation to BVOC emissions.

Due to CO_2/H_2O analyser malfunction, in some experiments data for photosynthesis rate was not reliable. For all experiments (where data for photosynthesis rates were reliable), net photosynthesis showed strong relationship to transpiration rates when the plants were exposed to drought. At PPFD = 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a chamber temperature of 20 °C both rates were correlated at $R^2 > 0.9$ (Figure 8), indicating that either of them could be used mutually as a reference basis. As transpiration data were reliably obtained for all experiments, I used transpiration data to characterize the degree of drought stress.

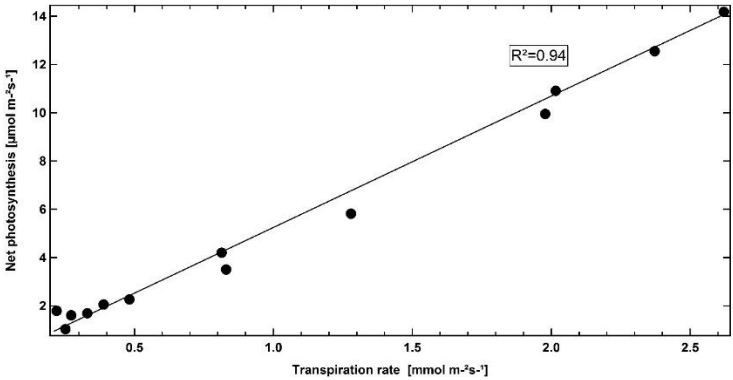


Figure 8 - Relationship between net photosynthesis and transpiration from tomato plant under diurnal light settings and drought. Only data at PAR = 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a chamber temperature of 20 °C are shown. Measurements were performed using CO_2/H_2O analyser.

4.3.2 Emissions from plants not exposed to MeJA

In all control plants, emissions of two compounds were most dominant – β-phellandrene and homoterpene TMTT. SQT emissions were very low and their concentrations were close to detection limit of the analytical device (~ 1 ppt). Except constitutive MT, TMTT and the minor amounts of β-caryophyllene, no other compounds were detected in control plants

In all experiments, drought stressed plants and controls, TMTT emissions started several hours after introducing a plant in a measuring chamber. TMTT emissions steadily increased on time scales of days. This observation supported the assumptions that TMTT emissions were induced by a so far unidentified stress in the measuring chambers that developed on a time scale of days (compare Figure 11).

For easier comparison, the drought was characterised by separating three phases (Figure 9). The first period (Phase 1) was defined as the period without drought effect on transpiration or on the plant's phenotype. During this stage transpiration increased slowly and at the end of this stage it reached its

maximum (Table 4). Thereafter, transpiration dropped substantially for drought stressed plants while it still increased for control plants. Mostly after the third day of halted irrigation, transpiration started to decrease and plants showed visual symptoms of drought such as wilting of older leaves (Phase 2, see Figure 9). In the last period of such experiments (Phase 3), transpiration of drought stressed plants approached zero and almost lost its diurnal pattern. At this point plants looked severely affected by drought, and in some cases they even died shortly thereafter (see Figure 9). Control plants showed still slightly increasing transpiration.

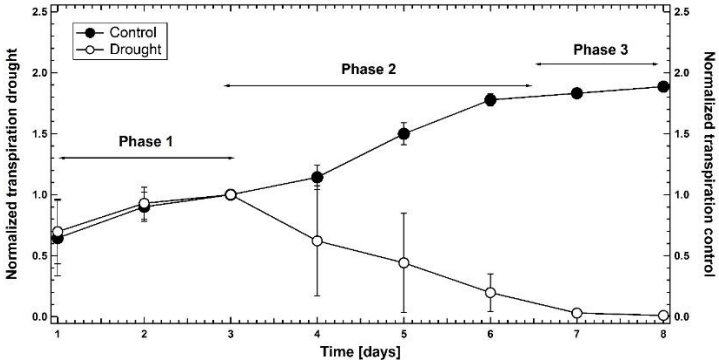


Figure 9 - Daily average transpiration rate of tomato plants under drought stress and controls under diurnal light settings in comparison with three drought phases. Data give the arithmetic mean and the standard error for five drought stressed and four control plants (Table 3) as averaged for the respective periods of illumination. For better comparison, transpiration data were normalized. Data for transpiration measured for a given plant at a certain time was divided by the average transpiration value of drought stressed plants (on the 3rd day).

The phases defined above also reflected the severity of visible symptoms of drought (Figure 10). During Phase 1, no symptoms of drought were observed. During Phase 2, older leaves started to wilt. At the end of Phase 3, plants were completely wilted.

Figure 10 - Visual symptoms of tomato plants in three drought phases; **A** - Phase 1, no visual symptoms of drought; **B** - Phase 2, older leaves starting to wilt; **C** - Phase 3, plant is completely wilted

4.3.3 Drought impact on TMTT, MT and GLV emissions under diurnal light rhythm

4.3.3.1 Drought impact on TMTT emissions

TMTT emissions showed distinct diurnal variations with increases after the light was switched on and decreases after the light was turned off. Maximum daily emissions were reached by the end of each day and emissions in the following nights were 20 to 30 % lower than the previous day maxima (Figure 11).

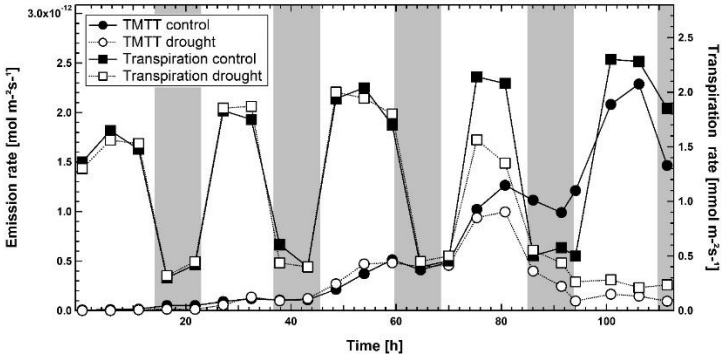


Figure 11 - TMTT and transpiration rate from a control and drought stressed tomato plant. Measurement was performed using CO_2/H_2O analyser parallel with GC-MS (sampling approximately every 320 min.). Shaded areas present night phases; TMTT - (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

TMTT emissions increased from day to day although temperature and light intensity were the same each day during the respective periods of illumination. Maxima for the TMTT emissions from control plants were reached on the last day of measurement (for quantitative data see Table 4).

During Phase 1, all BVOC emissions from drought stressed plants were similar to those of control plants: TMTT emissions showed diurnal variation with 20 – 30 % lower emissions in darkness and the emissions increased from day to day. Their maxima were found on day two or three i.e. at the end of Phase 1 (compare Figure 9).

When transpiration decreased during Phase 2, TMTT emissions also decreased but lagged several hours behind. During Phase 3, TMTT day and night emission rates were severely reduced (to below 7 % of their maxima reached at the end of Phase 1). At the point when transpiration lost any diurnal behaviour, TMTT emissions still showed some diurnal variation between light and dark phases (for quantitative data see Table 4).

Table 4 - Overview of average detected emission rates for the time period of 10-12 hours from five drought exposed plants and four control plants under diurnal light rhythm, without added methyl jasmonate; TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

Experiments without added MeJA – diurnal light rhythm	Average maximum emission rates from drought exposed plants	Average emissions from control plants detected at the time equivalent to the maximum emissions from drought exposed plants	Average maximum emission rates from control plants
Transpiration $mmol \cdot m^{-2} \cdot s^{-1}$	2.08 ± 0.4	2.1 ± 0.6	2.34 ± 0.5
TMTT $mol \cdot m^{-2} \cdot s^{-1}$	$(1.2 \pm 0.8) \cdot 10^{-12}$	$(1.3 \pm 0.7) \cdot 10^{-12}$	$(2.3 \pm 0.7) \cdot 10^{-12}$
α-terpinene $mol \cdot m^{-2} \cdot s^{-1}$	$(8.3 \pm 0.8) \cdot 10^{-13}$	$(2.6 \pm 0.8) \cdot 10^{-13}$	$(2.6 \pm 0.8) \cdot 10^{-13}$

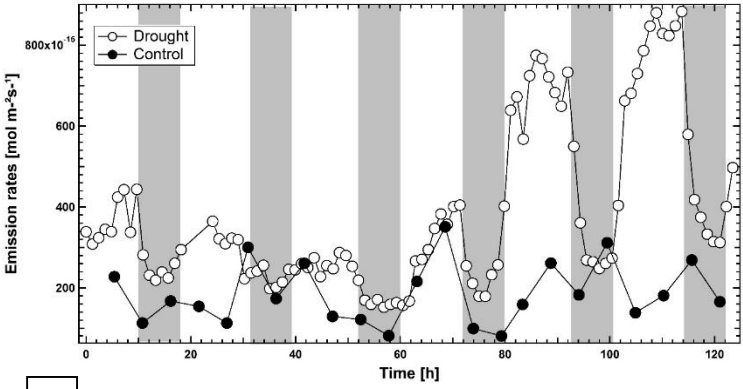
Re-watering a plant during the early Phase 3 led to recovery. Transpiration recovered much faster than TMTT emissions. Transpiration started to increase within one hour after irrigation while it took up to a whole day for a substantial increase of TMTT emissions (data not shown).

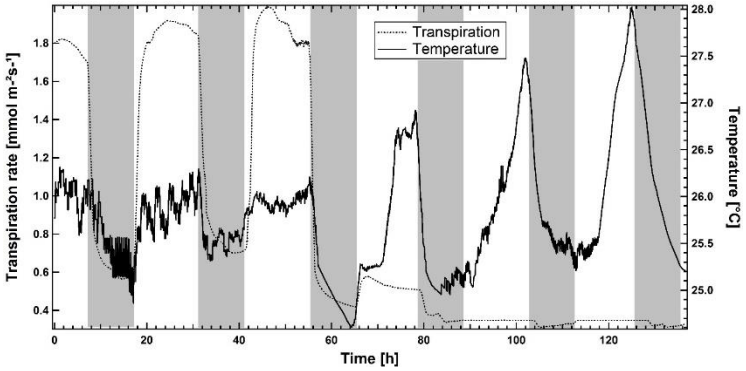
4.3.3.2 Drought impact on MT emissions

During phase 1, MT emissions showed marginal diurnal variation which in addition was superimposed by numerous small pulses. No significant differences were found between MT emissions from controls and from plants exposed to drought during this phase.

While no substantial changes of MT emissions from control plants were observed during the whole measurement period, MT emissions from drought stressed plants increased at the end of Phase 2. MT emissions increased about three fold and a substantial diurnal variation was observed from the end of Phase 2 when transpiration was already strongly suppressed (Figure 12a). Even after plants looked completely wilted and were apparently dead, MT emissions kept increasing for another 48 hours (for quantitative data see Table 4).

In order to investigate if the increase in MT emission during drought was related to changes of leaf temperature, I measured leaf temperature during drought. Although the temperature in the temperature housing was constant, leaf temperatures increased by 1-2° C during the development of severe drought due to limited transpiration (Figure 12B, compare Wu *et al.,* 2015).





B

Figure 12 - Impact of drought on monoterpene emissions from two drought stressed tomato plants; **A** - Time course of monoterpene emissions (α-terpinene) from first tomato plant under drought stress in comparison with well watered (control) plant. Measurements were performed using GC-MS with sampling every 70 min for the drought stressed plant and every 320 min for the control plant. Shaded areas present night phases; **B** - Leaf temperature compared to transpiration rate from second tomato plant under drought stress. Measurements were performed every two minutes. Shaded areas present night phases.

4.3.3.3 Drought impact on GLV emissions

GLV emissions were only detected during severe drought. Time frames for GLV releases differed from plant to plant. The earliest appearance of GLV emission was found when the transpiration rate had dropped to below 45 % of its maximum, the latest appearance was when transpiration had dropped to about 5 % of its maximum (data not shown). The dominant GLV was always (*Z*)-3-hexenol, and even from apparently dead plants, these emissions lasted for about 40 hours before they ceased. After that, the gas measurements went on for another two days. During that period, GLV emissions were no more detected, while MT emissions still kept increasing.

4.3.4 Drought impact on TMTT, MT and GLV emissions under permanent light

4.3.4.1 Drought impact on TMTT emissions

Experiments with diurnal variation of light had shown that, during severe drought, TMTT emissions lagged several hours behind the changes in transpiration. Furthermore, the steady increase in TMTT emissions during periods of illumination was interrupted by the dark phase. In order to look for the effects of drought without influence of the diurnal rhythm, these experiments were repeated with plants kept under permanent light.

Transpiration was quite constant from plant to plant. It either stabilized quickly or showed small increases within one day after placing the plants in the chamber. Control plants showed a rather continuous transpiration of a similar magnitude as drought stressed plants during Phase 1 (Table 5). In all plants exposed to drought, transpiration started to decrease between 35 and 45 hours after the last watering.

All plants showed TMTT emissions. After introducing a plant to the chamber, TMTT emissions increased steadily. Emissions from control plants reached a maximum at the end of the experiments (for quantitative data see Table 5). In drought stressed plants, TMTT emissions steadily increased until Phase 2 and thereafter decreased. For six out of seven drought stressed plants, the maxima of TMTT emissions were reached after transpiration was already reduced. In most cases, TMTT emissions were still growing after transpiration already had started to decrease (Figure 13), but eventually dropped to almost zero during Phase 3 (for quantitative data see Table 5).

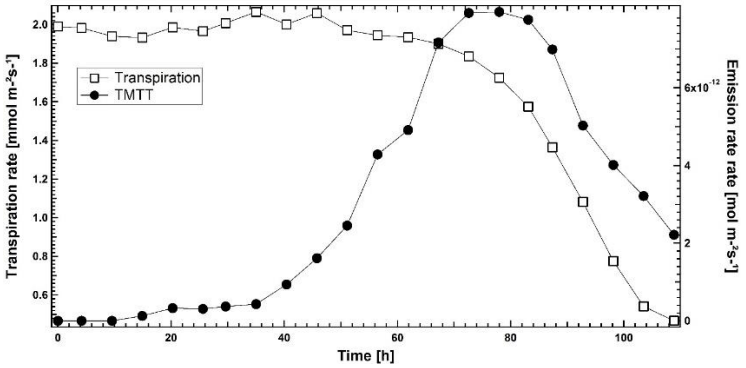


Figure 13 - Typical time courses of TMTT and transpiration rate from tomato plant exposed to drought stress and under constant light. TMTT measurement was performed using GC-MS analyser and gas sampling was taken every 320 min. Transpiration rate was measured with CO_2/H_2O analyser every 2 min and then averaged to match timing of GC-MS data**.** TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

All tested plants were watered just before starting the tests and showed no drought symptoms. In order to see if induction of TMTT emissions can be prevented by mild drought, a tomato plant with early visual drought symptoms was placed into the measuring chamber. Although transpiration of this plant was already decreasing from the beginning of the measurement, TMTT emissions increased over the next two days and in combination with the progressing drought, they gave a pattern very similar to the previously tested plants (Figure 14).

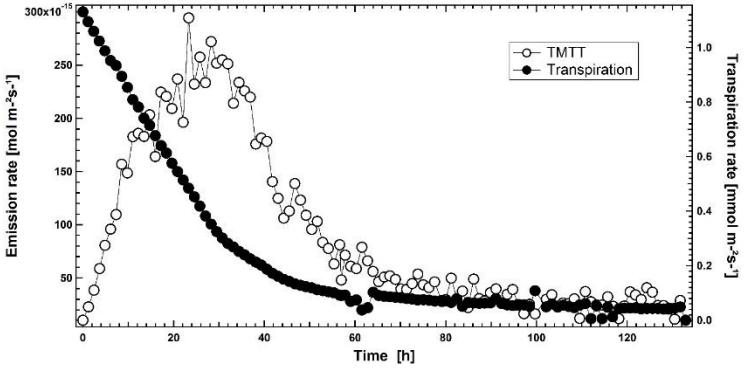


Figure 14 - Time courses of TMTT emissions and transpiration rate obtained for a tomato plant, which was placed into a chamber when there were already visible drought symptoms. TMTT measurement was performed by using GC-MS analyser and sampling was taken every 70 min. The transpiration rate was measured with CO_2/H_2O analyser every 2 min and then averaged for every hour**.** TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

4.3.4.2 Drought impacts on MT emissions

MT emissions of control plants showed minor fluctuations, but no substantial and systematic increases with time. MT emissions of drought stressed plants were quite constant during Phase 1 and the early Phase 2 but increased steadily after transpiration had dropped by 10 % to 60 %. MT releases were at their maxima near to the end of the experiments and were about four times higher than under stress free conditions (Table 5).

Table 5 - Overview of average detected emission rates for the time period of 10-12 hours from seven plants exposed to drought and four control plants under permanent light, without added methyl jasmonate; MeJA - methyl jasmonate, TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

Experiments without added MeJA - permanent light	Average maximum emission rates from drought exposed plants	Average emissions from control plants detected at the time equivalent to the maximum emissions from drought exposed plants	Average maximum emission rates from control plants
Transpiration $mmol \cdot m^{-2} \cdot s^{-1}$	2.0 ± 0.9	2.1 ± 0.4	2.1 ± 0.6
TMTT $mol \cdot m^{-2} \cdot s^{-1}$	$(4.5 \pm 1.3) \cdot 10^{-12}$	$(4.6 \pm 0.9) \cdot 10^{-12}$	$(8.3 \pm 0.7) \cdot 10^{-12}$
α-terpinene $mol \cdot m^{-2} \cdot s^{-1}$	$(1 \pm 0.8) \cdot 10^{-12}$	$(2.4 \pm 0.9) \cdot 10^{-13}$	$(2.5 \pm 0.8) \cdot 10^{-13}$

4.3.4.3 Drought impact on GLV emissions

No GLV emissions form control plants were detected. For six drought stressed plants, bursts of GLV release were observed when transpiration rate was severely reduced. Earliest bursts of GLV emissions were observed mid of Phase 2 when transpiration had dropped to below 40 %. GLV bursts appeared the latest, when transpiration had dropped to below 15 % of its respective maximum. Dominant GLV emission was that of (*Z*)-3-hexenol. Its maximum emission rates were different from plant to plant.

4.3.5 Drought impact on volatiles induced by methyl jasmonate exposure

MeJA exposure had no substantial impact on transpiration. For MeJA exposed as well as for non-exposed plants it took some time for transpiration to stabilize. Substantial differences in transpiration were only observed between the drought stressed and control plants, all of them exposed to MeJA.

No obvious impact of MeJA exposures on TMTT emissions nor on constitutive MT or GLV emissions was found. These emissions showed the same behaviour independent of the plants being exposed to MeJA or not.

In addition to the constitutively emitted MT and TMTT, all MeJA exposed plants emitted the MT (*E)*-β-ocimene and five HexD: *(Z)*-3-hexenyl propanoate, *(Z)*-3-hexenyl butyrate, *(Z)*-3-hexenyl isobutyrate, *(Z)*-3-hexenyl valerate and *(Z)*-3-hexenyl isovalerate. The appearance of these emissions was independent of light settings (permanent light and diurnal light rhythm). These emissions were not found for plants not exposed to MeJA and not after plant crushing.

4.3.5.1 Drought impact on (*E*)-β-ocimene emissions

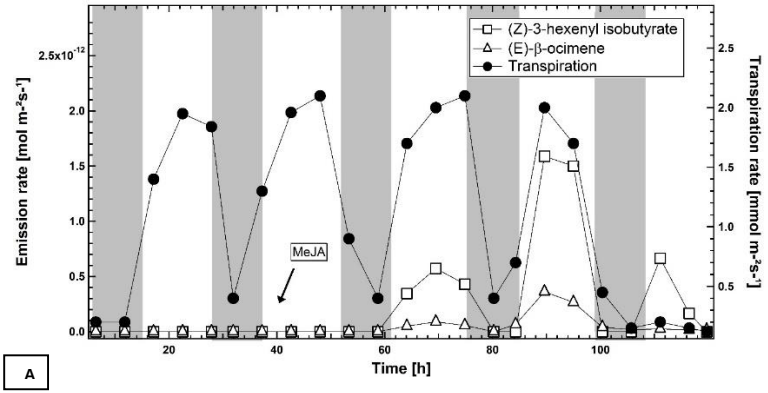
(*E*)-β-ocimene emissions appeared 14 to 20 hours after starting MeJA exposures. These emissions were not correlated with other MT emissions and showed a distinct diurnal behaviour with very low emissions during darkness. Similar to TMTT emissions, (*E*)-β-ocimene emission rates of drought stressed plants, under day and night light settings, increased with time and reached flat maxima on the second day after starting MeJA exposures (Figure 15A).

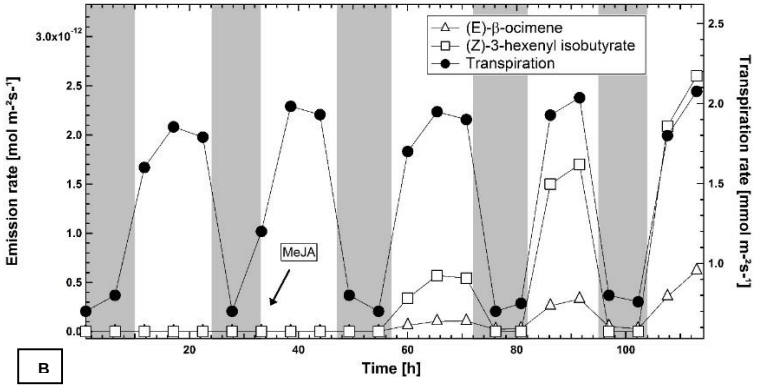
During Phase 1, the emissions from control and drought treated plants were similar (Figure 15A and 15B, for quantitative data see Table 6).

Table 6 - Overview of average induced emission rates for the time period of 10-12 hours from four plants exposed drought and five control plants, all plants were exposed to methyl jasmonate under diurnal light rhythm; MeJA - methyl jasmonate, TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

Experiments with added MeJA - diurnal light rhythm	Average maximum emission rates from drought exposed plants	Average emissions from control plants detected at the time equivalent to maximum emissions from drought exposed plants	Average maximum emission rates from control plants
***(E)*-β-ocimene** $mol \cdot m^{-2} \cdot s^{-1}$	$(3.2 \pm 1) \cdot 10^{-13}$	$(3.3 \pm 0.9) \cdot 10^{-13}$	$(4.2 \pm 0.8) \cdot 10^{-13}$
***(Z)*-3-hexenyl isobutyrate** $mol \cdot m^{-2} \cdot s^{-1}$	$(1.7 \pm 0.8) \cdot 10^{-12}$	$(1.8 \pm 0.9) \cdot 10^{-12}$	$(2.7 \pm 0.9) \cdot 10^{-12}$
TMTT $mol \cdot m^{-2} \cdot s^{-1}$	$(1.3 \pm 0.6) \cdot 10^{-12}$	$(1.4 \pm 0.7) \cdot 10^{-12}$	$(1.9 \pm 0.9) \cdot 10^{-12}$

During Phase 2, (*E*)-β-ocimene emissions from control plants increased further (~ 30 %). They reached their maxima at the end of the respective experiments. (*E*)-β-ocimene emissions from drought exposed plants dropped during Phase 2 coinciding with a lower transpiration (Figure 15A). These measurements were conducted at low time resolution (approximately five hours). Repeating the experiment with permanent light showed that there was a time lag between the decrease of transpiration and (*E*)-β-ocimene emissions (Figure 15C, Table 7).


(Z)-3-hexenyl isobutyrate
(E)-β-ocimene
Transpiration
MeJA
Emission rate [mol m-2s-1]
Transpiration rate [mmol m-2s-1]
Time [h]
2.5x10-12
A


(E)-β-ocimene
(Z)-3-hexenyl isobutyrate
Transpiration
MeJA
Emission rate [mol m-2s-1]
Transpiration rate [mmol m-2s-1]
Time [h]
3.0x10-12
B

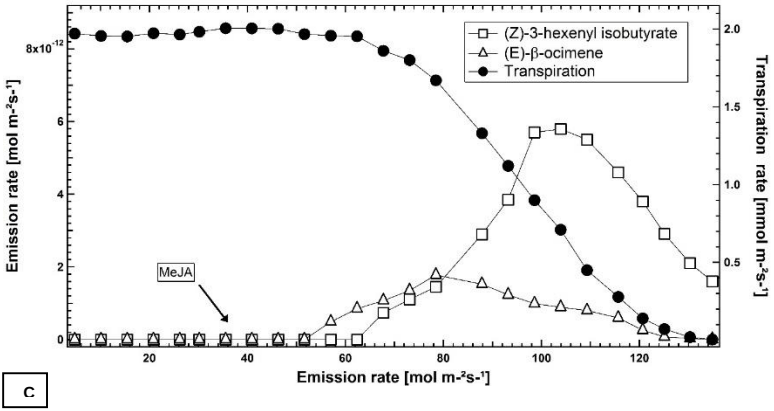


Figure 15 - Typical time courses of (*E*)-β-ocimene, (*Z*)-3-hexenyl isobutyrate emissions and transpiration from tomato; **A** - drought stress with diurnal light settings; **B** - control plant with diurnal light settings; **C** - drought stress with constant light settings. BVOC were measured by GC-MS analyser and sampled approximately every 320 min. Transpiration rates were measured with a CO_2/H_2O analyser. The arrow points at the time when a filter paper soaked with 0.05 ml methyl jasmonate was added into the measuring chamber.

4.3.5.2 Drought impact on emissions of hexenyl derivatives

All MeJA treated plants emitted HexD, regardless of light settings or drought exposure. Emissions of individual HexD were always correlated to each other ($R^2 > 0.94$) but no correlation was observed between the HexD emissions and the GLV that were also emitted by drought stressed plants. HexD emissions appeared much earlier than GLV emissions.

Dominant HexD was (*Z*)-3-hexenyl isobutyrate and quantitative data are given only for this compound. Similar to (*E*)-β-ocimene emissions, emissions of (*Z*)-3-hexenyl isobutyrate increased with time and they were strong during phases of illumination and nearly absent in darkness. Maximum emissions from control plants were observed at the end of the experiments (average maximum in Table 6, Figure 15B), whereas for drought stressed plants the maxima were reached at the early Phase 2 (Figure 15A). During

Phase 1, emissions from controls were similar to those from plants later exposed to drought (maximum on the 2nd day, Table 6).

Under constant light, emissions of (*Z*)-3-hexenyl isobutyrate from drought exposed plants compared to transpiration rate remained high for a longer period of time, but eventually they also decreased at a later stage (Figure 15A and 15C). Experiments with constant light also showed a longer delay of HexD emissions compared to transpiration or (*E*)-β-ocimene emissions (Table 7).

Compared to the drought-induced decreases of transpiration and net photosynthesis, the plants' reactions with respect to BVOC emissions appeared with time lags. These time lags were determined quantitatively by using the points in time when transpiration and BVOC emissions, respectively, had decreased by 50 % from their respective maxima. Lag periods were calculated for experiments conducted under permanent light and are listed in Table 7.

Table 7 - Overview of time delay between 50 % drop of maximum transpiration and 50 % drop of maximum emission rates, measured under constant light settings; MeJA - methyl jasmonate, TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

Emitted compound	Experiments with MeJA - delay in hours (four plants)	Experiments without MeJA - delay in hours (seven plants)
TMTT	34 ± 15.2	24.3 ± 9.9
(*E*)-β-ocimene	14.8 ± 6.6	-
(*Z*)-3-hexenyl isobutyrate	47.5 ± 23.75	-

4.4 Discussion

An increase in transpiration, which was observed in all tested plants during Phase 1, can be explained by plant adaptation to the measuring chamber, an increase in leaf area that was not considered when calculating transpiration, or recovery from possible overwatering. However, in both drought stressed and control plants, the same increases were observed and the plants had identical growing conditions before and during gas measurements. Therefore, no severe impact of early transpiration increases during Phase 1 on drought results were expected.

4.4.1 Impact of severe drought

Impacts of drought on constitutive emissions are described in literature (e.g. Sharkey and Loreto, 1993; Pegoraro *et al.,* 2004a; 2004b; Brilli *et al.,* 2007; Lehning *et al.,* 1999; Bertin and Staudt, 1996; Llusià and Peñuelas, 1998; Plaza *et al.,* 2005; Lavoir *et al.,* 2009; Šimpraga *et al.,* 2011; Bourtsoukidis *et al.,* 2014; Wu *et al.,* 2015). A general statement from all these reports is that constitutive BVOC emissions decrease when drought becomes severe.

In case of TMTT, (*E*)-β-ocimene, and HexD emissions ceased during severe drought (Phase 3) indicating that the response of these induced emissions is similar to that of constitutive emissions. Accordingly, these decreases were attributed to a general decrease of the plants' metabolism as it was also assumed for decreases of isoprene emissions (e.g. Brüggemann and Schnitzler, 2002) and for the constitutive *de-novo* MT emissions (Wu *et al.,* 2015).

Under severe drought, emissions of isoprene are decoupled from photosynthesis. This has been explained by the use of alternative carbon sources for isoprene biosynthesis (Possell and Loreto, 2013 and references cited therein). Such decoupling was also observed by Wu *et al.* (2015), who showed that decreases of constitutive *de-novo* MT emissions appear later than decreases of transpiration or net photosynthesis. The same behaviour was found here: TMTT, (*E*)-β-ocimene and HexD emissions decreased later and at higher levels of drought than transpiration and net-photosynthesis. It is assumed that the use of alternative carbon sources is one reason for the delay between the responses of the above mentioned induced emissions and net

photosynthesis. This assumption will be discussed at the example of TMTT emissions, as information on such alternative sources were obtained from a labelling experiment with tomato, conducted before beginning of this study.

Emissions of TMTT are *de-novo* emissions (Farag and Paré, 2002; Thaler *et al.,* 1996) and storage organs for TMTT have not been found in tomato plants (Ament *et al.,* 2006). Consistently, crushing of plant did not induce TMTT emissions. Exposing a tomato that emitted TMTT to $^{13}CO_2$ led to a fast incorporation of the ^{13}C into the emitted TMTT but the degree of labelling levelled out at roughly 66 % although the plant was exposed with $^{13}CO_2$ for several hours after the labelling had reached a steady state. Accordingly, roughly, one third of the TMTT must have been synthesized from another carbon source than from the carbon taken up via photosynthesis (personal communication with Dr. Jürgen Wildt). These alternative sources may deliver carbon, when the drought already suppressed the net photosynthesis.

Maintained TMTT synthesis, at strongly suppressed net photosynthesis, also requires that the enzymes responsible for TMTT synthesis are more tolerant to drought than the enzymes controlling CO_2 uptake. Such high drought tolerance has been shown for MT synthases (Grote *et al.,* 2010). Hence, high drought tolerance may also be given for the enzymes responsible for TMTT biosynthesis.

In total, the assumption of alternative carbon sources being responsible for the later response of TMTT emissions to the drought can explain the observed behaviour and is consistent to findings reported in literature. However, here the main focus lies on induced emissions and not on constitutive emissions. While constitutive emissions are mainly determined by temperature, light intensity and soil moisture, the strength of induced emissions may also depend on the effectivity of the elicitor inducing the respective emission. Therefore, it cannot be excluded that an increasing efficiency of the elicitor or a late response of activated signalling pathways with progressing drought are another reasons for the delayed response of TMTT emissions to the severe drought.

During Phase 3 of the drought, also the MeJA induced emissions of (*E*)-β-ocimene and the HexD decreased to almost zero. During all experiments, the (*E*)-β-ocimene emissions decreased earlier and already at lower degree of drought than the HexD emissions. Hence, differently induced emissions may show different temporal behaviour although the elicitor is the same. Reason for this may be that enzymes in their synthesis have different drought resistance or that alternative carbon sources are not identical for the synthesis of all induced BVOC. However, for all these emissions the response to drought appeared later than the responses in transpiration and net photosynthesis.

Besides decreasing emissions of TMTT, (*E*)-β-ocimene, and the HexD, increasing emissions of MT and GLV have been found. For MT this is the opposite behaviour than that described by Wu *et al.* (2015) who found ceasing emissions during a comparable phase of drought.

The reason for the different behaviour is the different basic emission mechanism for constitutive MT emissions from tomato and from the plants investigated by Wu *et al.* (2015). Wu *et al.* (2015) investigated *de-novo* emissions, while in the present study pool MT emissions were investigated. The increases observed here were at least three fold. Such strong increases cannot be explained by drought-induced increases of leaf temperatures. Assuming typical temperature coefficients of 0.09 to 0.12 K^{-1} (e.g. Kesselmeier and Staudt, 1999; Guenther *et al.,* 2006; 2012), three-fold increases would require increases of leaf temperature in the range of 9 °C, much higher than measured in my experiments with tomato (1 – 2 °C). I assume that the increases of MT emissions were due to mechanical damage of trichomes because wilting of leaves under drought can destroy trichomes. Such increases of MT emissions from tomato were also reported as consequence of necrosis (Jansen *et al*., 2009a) or heat stress (Copolovici *et al.,* 2012).

Increases of MT emissions due to wilting induced trichome damage appeared before GLV emissions started. GLV are produced and released within minutes after mechanical injury or herbivore feeding (Loreto *et al.,* 2006; Fall *et al.,* 1999). Their absence thus indicates that membrane damage in plants was not substantial when MT emissions increased. Obviously, early wilting caused trichome damage but not necessarily membrane damage. Since GLV emissions are metabolically synthesized and trichomes composed of dead plant matter, trichome destruction by wilting does not cause GLV emissions. I assume that the observed effect of increased MT emissions is just a physical and not a metabolically driven process.

Emissions of GLV are induced when membranes are damaged and the emissions are independent of the kind of stress inducing membrane damage (Heiden *et al.,* 2003). Hence, if severe drought induces membrane damage, GLV are released (Capitani *et al.,* 2009; Šimpraga *et al.,* 2011). Consistent for all these GLV emissions was that drought had to exceed a certain level of severity before the GLV were released. This was indicated by the late appearance of GLV emissions, which were not observed before the end of the drought Phase 2.

GLV emissions stopped at stages when MT were still released and plants might have been dead. Severe drought can suppress enzymatic steps

of the octadecanoid pathway; the physical process of MT evaporation can still continue when a plant is dead.

4.4.2 Impact of moderate drought

While a general decrease of constitutive *de-novo* BVOC emissions under severe drought is non-controversial, less agreement exists for moderate drought. There are reports on no changes at all compared to well-watered conditions as well as reports on substantial increases that may be up to three fold (Ormeño *et al.,* 2007). For TMTT, (*E*)-β-ocimene, and the HexD substantially increasing emissions during moderate drought (Phase 2) was also found, however, these increases are not ascribed to a direct impact of drought.

I assume that the temporal shape of the increases of (*E*)-β-ocimene and HexD was determined by the time needed to develop the effects of MeJA exposure from starting it until full development. During Phase 1 and Phase 2, the temporal shapes of these increases measured for drought stressed plants were similar to those measured for control plants. From this similarity, I conclude that the effects of mild stress in the process of inducing the emissions were minor. Only when the drought stress became severe, the differences became obvious by a decrease of emissions.

Similarly, from the nearly identical temporal shapes observed for drought stressed plants during Phases 1 and 2, and control plants, respectively, I conclude that the increase observed for TMTT emission was not induced by moderate drought.

Until now, there are no literature data available on the dynamic behaviour of stress induced emissions with progressing drought.

Gouinguene and Turlings (2002) measured the impact of abiotic factors on stress-induced emissions for plants growing at different soil moisture. Hence, the dynamics of the emissions such as increases in emissions due to stress intensity were not studied. Nevertheless, comparing the data obtained from different individuals, Gouinguene and Turlings (2002) found higher emissions for injured plants when growing at relative soil humidity of 20 – 40 % than for injured plants when growing at relative soil humidity 80 – 100 %. According to the relative soil humidity of 20 – 40 % the data during drought are comparable to the Phase 2 defined here. Compared to well watered conditions also here higher emissions have been found but, as mentioned above, the higher emissions are not attributed to the drought.

4.5 Summary and conclusions

My findings suggest that the general response of the induced emissions of TMTT, (*E*)-β-ocimene, and the HexD to drought is qualitatively similar to that of constitutive *de-novo* emissions. For plants growing without water deficit or at moderate degrees of drought there were intermittent increases. However, as obvious from the similar behaviour observed for drought stressed and control plants, these intermittent increases were no direct impact of drought. With increasing severity of drought, the emissions were suppressed. Compared to the plants' responses in transpiration and net photosynthesis, the responses in emissions appeared with a delay. One explanation for the time lag between decreasing emissions and decreasing net photosynthesis is use of alternative carbon sources. However, eventually the emissions cease.

Within this study, no induced BVOC emissions were found that could be related as a drought specific.

If stress induced emissions such as TMTT, (*E*)-β-ocimene, and the HexD play a role for plant communication with other organisms (Dicke, 2009; Arimura, 2005; Pickett *et al.,* 2003) their suppression by drought would as well interfere with plant – plant communication. As climate change may induce more and longer lasting drought periods (Dai, 2013), plant communication may be impeded in future.

5 IMPACT OF MILD OR EARLY BIOTIC STRESS ON BVOC EMISSIONS FROM TOMATO

5.1 Introduction

Mild (and also early) stress is hard to investigate by using BVOC emissions because it is usually associated with only minor changes in the BVOC blend (Niinemets *et al.,* 2013). Nevertheless, changes in BVOC emissions in early or mild stages of biotic stress can have advantages for the practical approach such as in plant phenotyping and early stress detection.

In this chapter, I describe the results obtained with some of the most common biotic stresses in tomato greenhouses such as grey mould (*Botrytis cinerea*), powdery mildew *(Oidium neolycopersici)*, aphid (*Myzus persicae*) and whitefly (*Trialeurodes vaporariorum*). These stresses were chosen because they are most common sources of economically important yield loss in greenhouses every year (personal communication with Dr. Jantineke Hofland-Zijlstra). Additionally, I investigated if changes of BVOC emissions, together with visual symptoms, can provide further information about the plants reactions to stress.

5.2 Specific materials and methods

An overview of all biotic stress experiments is presented in Table 8. and Table 9.

Table 8 - Overview of grey mould and powdery mildew experiments; GLV - green leaf volatiles, MT - monoterpenes, SQT - sesquiterpenes, TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, MeSA - methyl salicylate

Type of biotic stress		Number of tested plants	Age of plants	Length of stress exposure before measurement	Light settings	Detected BVOC
Grey mould *Botrytis cinerea*	Treatment	6	4 weeks	18 hours	Diurnal settings	GLV, MT, TMTT, MeSA, SQT
	Control	6	4 weeks	-		MT, TMTT
Powdery mildew *Neodium lycopersici*	Treatment	6	4 weeks	2 weeks	Constant light	MT, TMTT, MeSA, SQT
	Control	6	4 weeks	-		MT, TMTT

Table 9 - Overview of aphid and whitefly experiments; GLV - green leaf volatiles, MT - monoterpenes, SQT - sesquiterpenes, TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, MeSA - methyl salicylate, BA - benzoic acid

Type of biotic stress		Number of tested plants	Age of plants	Length of stress exposure before measurement	Light settings	Detected BVOC
Aphid *Myzus persicae*	Treatment	6	4 weeks	2 weeks	Diurnal settings	MT, TMTT, MeSA, SQT
			6 weeks	4 weeks		MT, TMTT, MeSA, SQT
	Control	6	5 weeks	-		MT, TMTT
Whitefly *Trialeurodes vaporariorum*	Ttreatment	6	4 weeks	2 weeks	Diurnal settings	MT, TMTT, MeSA, SQT, BA
	Control	4	4 weeks	-		

5.2.1 Grey mould (*Botrytis cinerea*)

Grey mould (GM) inoculation solution was prepared according to Jansen *et al.* (2009a). *Botrytis cinerea* strain B0510 was growing on Malt Extract Agar (CM0059, Oxoid, BASTINGSSTROKE, UK) in concentration of 50 g per L. The culture was growing in petridishes and it was incubated in darkness at 20 °C until the mycelium had reached edges of the petridish (3-5 days). After that, the petridishes were exposed for 2-3 days to normal daylight and thereafter returned to darkness. In the following days, the culture turned from white to clearly grey indicating timing for spore harvest.

The final spore solution contained spores in concentration $1 \cdot 10^6 \cdot ml^{-1}$ in Potato Dextrose Broth (12 $g \cdot l^{-1,}$ Difco, USA). Six tomato plants were inoculated at the stage of four weeks by spraying each plant with about 15 ml of inoculation solution. Thereafter plants were placed in the measuring chambers with relative humidity near to 100 % and in darkness for a period of

18 hours (Table 10). Before starting BVOC measurements, illumination and air humidity in the chamber were adjusted to standard settings (described in Chapter 2). Each experiment lasted four days and during that period, plants were daily watered. Control plants were prepared in the same way as GM infected plants, but GM spores were replaced with water.

Table 10 - Experimental set-up for grey mould infection and measurement schedule with chamber settings; PPFD - photosynthetic photon flux density, GC-MS - gas chromatography mass spectrometry

Phase	Period (h) after inoculation		Humidity (%)	PPFD $\mu mol \cdot m^{-2} \cdot s^{-1}$	GC-MS measurement
	Start	End			
Dark phase	0	18	100	0	No
Day 1	18	32	~75	400	Yes
Night 1	32	42	~20	0	Yes
Day 2	42	56	~75	400	Yes
Night 2	56	66	~20	0	Yes
Day 3	66	80	~75	400	Yes
Night 3	80	90	~20	0	Yes
Day 4	90	104	~75	400	Yes

5.2.2 Powdery mildew (*Oidium neolycopersici*)

Powdery mildew (PM) infected plants were collected directly from an experimental greenhouse (located in Wageningen University and Research Centre) and introduced into a glass made growing chamber containing about 30 healthy tomato plants. After PM had spread on all 30 plants inside of the PM

growing chamber, additional six healthy plants (later used for BVOC measurements) were introduced into the PM growing chamber.

Plants used for BVOC testing were two weeks old when they were introduced into the PM growing chamber. Gas emissions were measured at the plant stage of four weeks, when first visual symptoms were observed. Plants were introduced into the measuring chamber 24 hours before starting BVOC measurement. For each plant, total BVOC measurements lasted approximately two days. Within that time, GC-MS measurements were conducted every 320 min. Gas sampling for each measuring point lasted about 50 minutes. Control plants were grown under identical growing conditions, just without PM presence.

5.2.3 Aphid (*Myzus persicae*)

Aphid individuals were collected directly from experimental greenhouse located in the Department of Molecular Phytomedicine, INRES, University Bonn. They were first raised in a plant growth chamber on kale seedlings, and then manually transferred on six plants at the stage of two weeks, about 100 individuals per plant. At the stage of four weeks, tomato plants together with the aphids were introduced into a BVOC measuring chamber for an early stress gas measurement. After gas measurement, plants were placed back into the growth chamber. During this process, plants were handled very carefully in order to prevent aphids to fall off the leaves. At the stage of six weeks, the same plants were reintroduced into the BVOC measuring chamber and tested for BVOC changes after longer aphid exposure.

Six control plants were grown under identical conditions as stressed plants just without aphids. Emission rates from control plants were measured only at the stage of five weeks since changes in constitutive emissions between two measurements were not expected. After introducing plants into a measuring chamber, they were left for about 24 hours to adapt to chamber light and temperature settings. BVOC testing lasted one day per plant. In order to avoid any changes in aphid behaviour, these tests were conducted under diurnal light settings. The actual number of aphid nymphs was not counted since handling of plants was kept at a minimum in order to prevent aphids to fall off the plants. The approximate number of aphid individuals was estimated by photographing and then counting aphids from randomly selected 10 infested tomato leaflets.

5.2.4 Whitefly (*Trialeurodes vaporariorum*)

Six healthy two weeks old tomato plants were introduced into a WF breading cage with a volume of about 1 m^3 and located in a greenhouse at the Department of Molecular Phytomedicine, INRES, University Bonn. Plants were exposed to natural day and night variations and temperatures (15th August – 30th August). The cage contained about 1000 virus free WF individuals and two already infected tomato plants. At the stage of four weeks, infested plants were introduced into the BVOC measuring chamber. About 50 WF were placed on each individual plant. BVOC emissions from WF infested plants were first measured by JPAC GC-MS system after 50 minutes gas sampling. Peaks in these chromatograms were not high enough for precise determination of BVOC mixing ratios. Therefore, BVOC emissions from WF infected plants were collected by 24 hours off-line gas sampling. These samples were then tested by the second GC-MS system placed in Wageningen University and Research Centre (see Chapter 2).

5.3 Results

5.3.1 Grey mould (*Botrytis cinerea*)

Plants infected with grey mould (GM) started to develop visual symptoms after the first dark phase after plant inoculation (Table 10). By the end of the first day, symptoms were most obvious. The most common symptoms were numerous small dark spots, developed directly under droplets of GM inoculation solution, and some larger necrotic spots surrounded by dark edges (Figure 16). Visual symptoms did not vary much between plants, but because of the tininess and high number of necrotic spots, it was impossible specify leaf area covered with necrotic spots. On the first day after plant inoculation, necrotic spots development was quite fast and very clear. However, it seemed that, in the following days, the development of necrotic spots slowed down or even completely stopped. In the following two weeks, plants continued to grow healthy new leaves. Depending on the severity of injury, injured leaves either continued to grow or dried out and fell off.

Figure 16 - Visual symptoms of grey mould infection on a tomato plant.

All detected BVOC and their average emission rates from GM infected plants and controls are presented in Table 11. Emission rates showed a high variability from plant to plant, but some general behaviour was found. Major emissions detected from control plants were the constitutive MT emissions and TMTT. β-caryophyllene was the only SQT where some emissions were detected, but the emission rates were too low to be quantified. (*E*)-β-ocimene, GLV, HexD or MeSA emissions from control plants were not detectable.

Table 11 - Average emission rates (mol·m^{-2}·s^{-1}) for daily illumination time period (14 hours), with standard error, detected from six grey mould infected plants and compared to six control plants; GM - grey mould, TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, MeSA - methyl salicylate. Statistical difference between grey mould infested plants and controls was calculate according to T-test, * P< 0.05, ** P< 0.01, - - below detection limit and below 10^{-16} mol·m^{-2}·s^{-1}

Detected compounds	DAY 1		DAY 2		DAY 3		DAY 4	
	GM	Control	GM	Control	GM	Control	GM	Control
(*Z*)-3-hexenol	$(1.2 \pm 0.5) \cdot 10^{-9}$**	-	$(1.1 \pm 0.4) \cdot 10^{-11}$**	-	$(4.4 \pm 1.8) \cdot 10^{-12}$**	-	-	-
α-terpinene	$(1.5 \pm 0.6) \cdot 10^{-11}$**	$(3 \pm 1.5) \cdot 10^{-13}$	$(4 \pm 1.6) \cdot 10^{-12}$**	$(2.9 \pm 1.4) \cdot 10^{-13}$	$(1.5 \pm 0.6) \cdot 10^{-12}$**	$(4.2 \pm 2.1) \cdot 10^{-13}$	$(8.5 \pm 1.7) \cdot 10^{-13}$*	$(4.3 \pm 2.1) \cdot 10^{-13}$
(E)-β-ocimene	$(3.4 \pm 1.3) \cdot 10^{-13}$**	-	$(1.6 \pm 0.6) \cdot 10^{-13}$**	-	-	-	-	-
Aristolene	$(2.7 \pm 1.1) \cdot 10^{-14}$**	-	$(7.2 \pm 1.7) \cdot 10^{-15}$**	-	$(4.9 \pm 2) \cdot 10^{-15}$**	-	-	-
Valencene	$(5.3 \pm 2.1) \cdot 10^{-14}$**	-	$(4.4 \pm 1.8) \cdot 10^{-14}$**	-	$(5.1 \pm 2) \cdot 10^{-15}$**	-	-	-
α-copaene	$(1.3 \pm 0.5) \cdot 10^{-12}$**	-	$(3.1 \pm 1.2) \cdot 10^{-13}$**	-	$(7.6 \pm 3.1) \cdot 10^{-14}$**	-	$(3.2 \pm 2.2) \cdot 10^{-14}$**	-
δ-elemene	$(1.2 \pm 0.5) \cdot 10^{-14}$**	-	$(0.6 \pm 0.6) \cdot 10^{-14}$**	-	$(6.6 \pm 2.7) \cdot 10^{-15}$**	-	-	-

β-caryophyllene	**(2.3 ± 0.9) ·10^{-13}****	**-**	**(3.2 ± 1.3) ·10^{-14}****	**-**	**(1.5 ± 0.6) ·10^{-14}****	**-**	**-**	**-**
β-selinene	**(3.6 ± 1.4) ·10^{-14}****	**-**	**(2.4 ± 1.4) ·10^{-14}****	**-**	**(1.7 ± 0.7) ·10^{-15}****	**-**	**-**	**-**
MeSA	**(2.4 ± 0.9) ·10^{-14}****	**-**	**-**	**-**	**-**	**-**	**-**	**-**
TMTT	**(2.3 ± 0.4) ·10^{-14}****	**(1.4 ± 0.3) ·10^{-13}**	**(2.5 ± 1) ·10^{-14}****	**(6 ± 0.8) ·10^{-13}**	**(5.1 ± 2) ·10^{-14}****	**(1.8 ± 0.3) ·10^{-12}**	**(8.7 ± 6.2) ·10^{-14} ****	**(2.6 ± 0.5) ·10^{-12}**
(*Z*)-3-hexenyl butyrate	**(2.5 ± 0.7) ·10 $^{-12}$****	**-**	**(9.3 ± 5.3) ·10^{-13}****	**-**	**-**	**-**	**-**	**-**
(*Z*)-3-hexenyl propanoate	**(4.9 ± 1.8) ·10^{-13}****	**-**	**(7.4 ± 4.3) ·10^{-14}****	**-**	**-**	**-**	**-**	**-**
(*Z*)-3-hexenyl valerate	**(1.2 ± 0.7) ·10^{-13}****	**-**	**(1.7 ± 1) ·10^{-14}****	**-**	**-**	**-**	**-**	**-**
(Z)-3-hexenyl isobutyrate	**(3.8 ± 1.2) ·10^{-12}****	**-**	**(1.2 ± 0.7) ·10^{-13}****	**-**	**-**	**-**	**-**	**-**

GM infected plants emitted the same compounds as the control plants with addition of the induced MT (*E*)-β-ocimene, several SQT, GLV, HexD and MeSA. The first day after infestation, emission rates of GLV and the

constitutively emitted MT were the highest in GM infected plants. Thereafter these emissions decreased from day to day. α-terpinene emissions from GM infected plants on the first day were by factor of 50 higher than emissions of control plants (Table 11).

(E)-β-ocimene emissions showed a strong diurnal modulation with high emissions during periods of illumination and no emissions during darkness. Emissions varied from plant to plant. Highest emissions were detected on the first day after infection. Thereafter emissions decreased from day to day. Three days after the infection, *(E)*-β-ocimene emissions were below the detection limit of the analytical device (Table 11).

SQT emitted from GM infected plants were aristolene, valencene, α-copaene, δ- elemene, β-selinene and β-caryophyllene. SQT emissions also showed a diurnal modulation with light and decreasing emission rates from day to day with the highest emissions on the day one. Mainly emitted SQT were α-copaene (74 % of all SQT) and β-caryophyllene (13 % of all SQT). Emission rates of α-copaene and β-caryophyllene were not correlated. The rest of the SQT emissions were correlated with β-caryophyllene, therefore the focus was mainly on β-caryophyllene and α-copaene. By the day four after infection, β-caryophyllene emissions were below the detection limit of the analytical device and emissions of α-copaene were still present. α-copaene emissions were detectable for another two weeks even at the stage when plants were almost fully recovered.

MeSA emissions from GM infected plants were detected only on the first day after infection. Compared to emissions of GLV or MT, MeSA emissions were quite low (Table 11).

TMTT emissions showed high variability between all tested plants. Average TMTT emissions from GM infected plants increased from day to day, but compared to controls, much slower. On the fourth day after treatment, control plants emitted about 22 times more TMTT than GM infected plants (Table 11).

HexD emissions were detected only in GM infected plants. As in tests with ozone and MeJA exposures, the emissions of (*Z*)-3-hexenyl butyrate, (*Z*)-3-hexenyl valerate, (*Z*)-3-hexenyl isobutyrate and (*Z*)-3-hexenyl propanoate were correlated (R^2>0.94, Figure 17). In contrast to GLV emissions, they showed diurnal behaviour. HexD emissions were strongest on the first day after GM infection. Already on the day 3 emission rates had dropped to below the detection limit of the analytical device (Table 11).

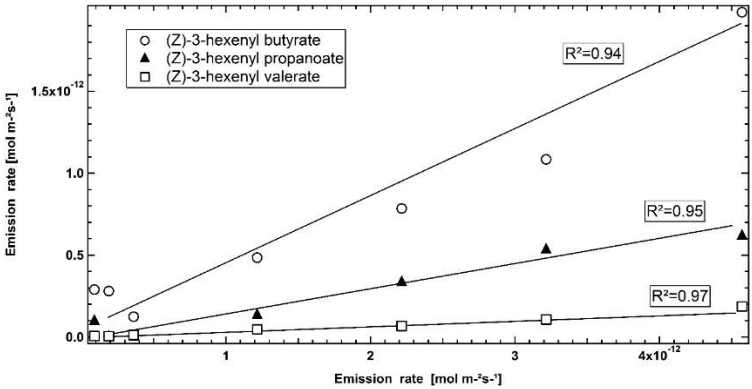


Figure 17 - Plot (*Z*)-3-hexenyl isobutyrate and other HexD emissions ((*Z*)-3-hexenyl butyrate, (*Z*)-3-hexenyl propanoate and (Z)-3-hexenyl valerate) from grey mould infected plant.

5.3.2 Powdery mildew (*Oidium neolycopersici*)

PM symptoms started as a chlorotic spots on the surface of plant leaves. Within few days these spots gradually turned into white powdery areas. At the stage of few weeks when plants were tested for BVOC emissions, old leaves were covered with clearly white areas. Newer leaves had either no symptoms at all or only symptoms of very early stages of infection (Figure 18).

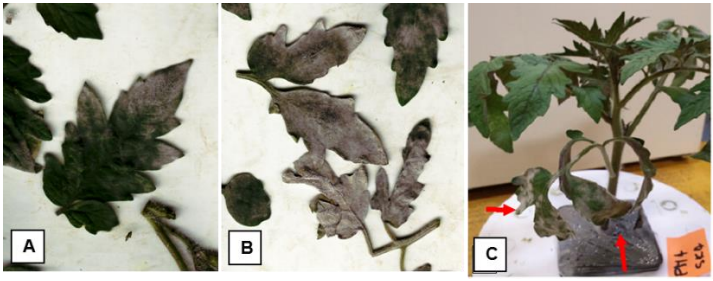


Figure 18 - Visual symptoms of powdery mildew infected tomato: **A** - mild infected leaf; **B** - severely infected leaves; **C** - tomato plant at the stage of four weeks, just before BVOC testing. Red arrows are pointing at infected leaves.

In order to test plants recovery, some plants were kept in the PM growing chamber for a longer period. In those plants, with the time, fungus had spread on all plant parts. Leaves with the longest infection dried out and fell off. Although there were some continuous growths of new leaves, the fungus eventually had killed the plants.

All tested plants including control and PM infected plants showed constitutive MT and TMTT emissions. PM infected plants additionally emitted the SQT δ-elemene, α- copaene and β-caryophyllene. Emissions of all three SQT were very low and they were not detectable in all chromatograms. Data on these SQT emission rates are not shown here. Only in one out of six plants, α-copaene emissions were always above detection limit of the analytical device. MeSA and GLV emissions were not detected at all (Table 12).

Table 12 - Average emission rates values for the 48 hour time period and standard errors for six powdery mildew infected plants compared to six control plants; TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, MeSA - methyl salicylate. Statistical difference between powdery mildew infected plants and controls was calculated according to T-test * P< 0.05, ** P< 0.01, $<10^{-16}$ - below detection limit and below $1 \cdot 10^{-16}$ mol·m^{-2}·s^{-1}

Detected compound	Powdery mildew mol·m^{-2}·s^{-1}	Control mol·m^{-2}·s^{-1}
(*Z*)-3-hexenol	$<10^{-16}$	$<10^{-16}$
α-terpinene	$(3.2 \pm 1.3) \cdot 10^{-13}$*	$(1 \pm 0.4) \cdot 10^{-13}$
α-copaene	$(3.8 \pm 1.5) \cdot 10^{-14}$**	$<10^{-16}$
β-caryophyllene	$(1.4 \pm 0.5) \cdot 10^{-14}$**	$(4 \pm 1.6) \cdot 10^{-15}$
TMTT	$(9.9 \pm 4) \cdot 10^{-13}$	$(1 \pm 0.4) \cdot 10^{-12}$
MeSA	$<10^{-16}$	$<10^{-16}$

Emission rates of α-terpinene and β-caryophyllene from PM infected plants were in average by a factor of three higher than in control plants. TMTT emissions of PM infected plants were similar to control plants. The only observable difference between BVOC patterns of controls and PM infected plants respectively was the presence of α-copaene in PM infected plants. α-copaene emissions were not detected in control plants (Table 12).

5.3.3 Aphids (*Myzus persicae*)

Aphids reproduced much slower on tomato plants than on kale seedlings. While kales were completely covered with aphid nymphs, the number of aphids on tomato plants almost stagnated at only about 30 per leaf. At the stage of two weeks after aphid infection, tomato plants showed no visual symptoms except of the presence of aphid themselves. After four weeks of aphid exposure, the number of aphid nymphs increased by about 50 % causing minimum leaf curling on few leaves and slightly stickiness of leaves due to aphid excretion (Figure 19). Typical visual symptoms of aphid infestation such as chlorosis, necrosis, wilting, and malformation of new growth (Goggin, 2007) were not observed.

Figure 19 - Tomato leaf after six weeks of aphid infection

Emission rates showed significant differences between aphid-infested plants and control plants. Besides the constitutive MT and the TMTT emissions, some aphid infested tomato plants also emitted β-caryophyllene, δ-elemene and α-copaene. In four out of six plants, α-copaene emissions were

detected but their emission rates were close to detection limit of analytical device. In plants where these emissions were observed, they were not always above detection limit. MeSA emissions were detected in five out of six plants. No significant SQT or MeSA emissions from control plants were detected.

After four weeks of aphid exposure, MeSA emissions were detected in all plants. SQT emissions were detected, but only in some plants and not in all chromatograms. No GLV emissions were found in any of the tested plants (aphid exposed or controls). There was also no significant difference between the emissions when measuring emissions from plants that were two weeks and four weeks under aphid exposure (Table 13).

Table 13 - Average day emission rates ($mol \cdot m^{-2} \cdot s^{-1}$) for the illumination time period (14 hours) and standard errors of six tomato plants after two and four weeks of aphid exposure and compared to controls; TMTT - (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, MeSA - methyl salicylate; statistical difference between aphid infested plants and controls was calculate according to T-test, ** P< 0.01

Detected compound	2 weeks exposure	4 weeks exposure	Control
α- terpinene $mol \cdot m^{-2} \cdot s^{-1}$	**$(6.4 \pm 1.4) \cdot 10^{-13}$****	**$(9.8 \pm 2.5) \cdot 10^{-13}$****	**$(9 \pm 2.5) \cdot 10^{-14}$**
MeSA $mol \cdot m^{-2} \cdot s^{-1}$	**$(2.1 \pm 0.8) \cdot 10^{-14}$****	**$(2.4 \pm 0.6) \cdot 10^{-14}$****	**$< 10^{-16}$**
TMTT $mol \cdot m^{-2} \cdot s^{-1}$	**$(1.6 \pm 0.6) \cdot 10^{-12}$****	**$(2.2 \pm 0.9) \cdot 10^{-12}$****	**$(9.7 \pm 3.8) \cdot 10^{-14}$**

Emission rates of TMTT and α-terpinene differed significantly between infested plants and control plants (Table 13). TMTT emissions of aphid-infested plants were in average 16 times (2 weeks exposure) and 22 times (4 weeks exposure) higher than from control plants. After two more weeks of aphid exposure, α-terpinene emissions were elevated. Compared to control plants, α-terpinene emissions were increased 7 fold after two weeks exposure and 11 fold after four weeks of aphid exposure (Table 13). MeSA emissions were detected only in aphid-infested plants, but the duration of the aphid exposure did not have any significant influence on its emission rates.

5.3.4 Whitefly (*Trialeurodes vaporariorum*)

WF infected plants showed no difference in visual appearance compared to control plants except the presence of flies on tomato leaves. No signs of substantial leaf damage such as chlorotic or necrotic lesions (Berlinger, 1986) were found (Figure 20).

Figure 20 - Tomato leaves after two weeks exposure to whitefly.

For all tested plants in WF experiments, all gas emissions were first sampled for 50 minutes and then analysed with the JPAC GC-MS system. In those samples, only constitutive emissions and TMTT emissions were above the detection limit. Therefore, emitted gases were sampled off-line for 24 hours, and analysed with a second GC-MS system placed in Wageningen University and Research Centre (see Chapter 2 - General methods). Unfortunately, this GC-MS system did not have a calibration system and emission rates could not be calculated. However, since all other parameters for calculating emission rates were known, it was possible to compare these data to data from control plants. Twenty-four hour sampling showed more compounds emitted by tomato plants than after 50 minutes sampling. Several different MT including β-myrcene, 2-carene and limonene oxide, and benzoic acid were observed when sampling for 24 h. The only detected SQT were β-phellandrene and α-copaene (Table 14).

Table 14 – Average detected BVOC emissions from six whitefly-infested plants and four controls for the 24-hours time period. Values are presented in arbitrary units (counts $m^{-2} \cdot s^{-1} \cdot 10^{10}$). Statistical difference between WF infested plants and controls was calculate according to T-test, ** P<0.01

Group of gasses	Detected compounds	Whitefly infested plants	Control plants
GLV	(*Z*)-3-hexenol	360 ± 120**	5.8 ± 3.3
Monoterpenes	α-terpinene	26.6 ± 8.8**	6.4 ± 3.4
	(*E*)-β-ocimene	3 ± 1.7	1 ± 0.5
Sesquiterpenes	α-copaene	124 ± 41**	7.9 ± 4.6
	β-caryophyllene	76 ± 25 **	6.4 ± 3.6
Homoterpenes	(*E,E*) - 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)	23 ± 3**	2.1 ± 1.4
Aromatic compounds	Methyl salicylate (MeSA)	163 ± 54**	15.2 ± 10.5
	Benzoic acid	122 ± 40 **	17.1 ± 9.87

All detected emissions from WF infected plants were also detected in control plants. Almost all emissions from WF infected plants were significantly higher except for *(E)*-β-ocimene, which were generally very low in all, WF infested and control plants (Table 14).

5.4 Discussion

5.4.1 Grey mould (*Botrytis cinerea)*

Botrytis cinerea is a fungal pathogen with necroptrophic lifestyle. It first kills host cells by secretion of toxins and lytic enzymes, what leads to a decomposition of plant tissue followed by consumption by the fungus (van Kan, 2006). Visual symptoms reflected failed GM infections (private communication with Dr. Jan van Kan). Just as in the previously described ozone study (Chapter 3), the results presented here implied that the development of visual symptoms and BVOC emissions were correlated. On the first day after infestation, necrotic spots developed fastest and in the same time, most intense changes in tomato BVOC emissions were observed. Later on, progress of necrotic spots either had slowed down or stopped, again followed by similar changes in BVOC emissions. Necrotic spots led to severe membrane damage and trichome damage (Jansen *et al.,* 2009a) resulting in bursts of GLV emissions and increases in MT emissions. Furthermore, SQT stored in trichomes such as β-caryophyllene and δ-elemene (Jansen *et al.,* 2009a; 2011; Schilmiller *et al.,* 2010) were also released in higher amounts due to necrotic spots development.

TMTT emitted by both control and GM infected plants continuously increased. However, in GM infected plants, the increase in TMTT emissions was much slower. Although reasons for this slower TMTT increase at this point are unknown, it seems that it was most likely caused by the presence of the fungus.

Emissions of α-copaene did not show any correlation to the emissions of the any other detected SQT such as aristolene, valencene, δ-elemene, β-selinene and β-caryophyllene. The emissions of α-copaene therefore seem to be increased due to another mechanism than an increased release from trichomes. α-copaene emissions seem to be directly induced by GM. This is in agreement with the findings of Thelen *et al.* (2005) who also observed that α-copaene emissions were induced by GM infection.

In previous experiments with MeJA exposures (Chapter 3), α-copaene emissions were detectable from MeJA treated plants. It is thus possible that α-copaene emissions from GM infected plants were also triggered via the JA pathway. However, high α-copaene emissions in GM infected plants were related to the presence of the parasite. In GM infected plants, α-copaene was detected even two weeks after infection, meaning that pathogen infection might have a long-term impact on α-copaene emissions.

Increased MeSA emissions indicate increased activation of the SA pathway. Since MeSA emissions during GM infection were limited to day one only, the activation of the SA pathway should have been attenuated during the later periods of these GM infections. This is consistent with results obtained by Thelen *et al.* (2005) who detected no significant MeSA emissions from tomato leaves on the second day after GM infection. However, this finding is in contradiction with a report from Jansen *et al.* (2009b) who showed long lasting and strong emissions of MeSA ($2 \cdot 10^{-10}$ mol·m^{-2}·s^{-1}) from GM infected tomato plants. The results of Jansen *et al*. (2009b) were obtained under identical conditions, with the same equipment as used here, including the identical GM strain. Obviously, tomato plants can show completely different reactions to GM infections. Strong differences were also observed for the visible symptoms. While the plants investigated by Jansen *et al*. (2009b) showed large fractions of dead plant material on the leaves after GM infestation, only minor areas with necrotic spots were observed here.

Jasmonate mediated pathways are activated in response to necrotrophic fungi such as GM (Glazebrook, 2005; Peña-Cortés *et al*., 2004; Antico *et al.,* 2012). Furthermore, MeJA exposures increase plant resistance against necrotrophic fungi species including GM (Zhu and Shiping*,* 2012; Yu *et al.,* 2009; Farmer and Ryan, 1992; El Oirdi *et al.,* 2011) and jasmonate deficient plants are more susceptible to GM (Díaz *et al.,* 2002). El Oirdi *et al.* (2011) furthermore showed that GM infestation can trigger different response in tomato plants. Activation of the SA pathway by GM can suppress the JA pathway, which subsequently can cause plant death rather than recovery (El Oirdi *et al.,* 2011). Such a process might explain the differences between the results of Jansen *et al*. (2009b) and those obtained here. The plants investigated here were capable to prevent further spreading of GM infection, possibly caused by an activation of the JA pathway. Consistently GM infected plants emitted (*E*)-β-ocimene, a BVOC that was also emitted during MeJA exposures.

The assumption of a successful defence by activation of the JA pathway is supported by differences in observations made for the BVOC emissions in the experiments of Jansen *et al.* (2009b) who did not observe (*E*)-β-ocimene emissions from GM infested plants. This may indicate that the JA pathway was induced here but not in the experiments of Jansen *et al.* (2009b).

In here presented results, MeSA emissions were present only shortly after infection, showing that plant had SA pathway activated most likely only during the spreading of necrotic spots. Probably it took several hours for the plants to adapt to GM infection, to induce the JA pathway and to stop further developments of necrotic spots.

It seems that in my experiments it took about one day for the plants to overcome GM and prevent the growth of necrotic spots. During this period, most BVOC emissions were detected. After this stage, emission rates were slowly recovering to the level observed for control plants. This behaviour might indicate successful plant adoption to pathogen and a fine-tuning between plant defence mechanisms (for a review see Derksen *et al.,* 2013).

5.4.2 Powdery mildew (*Oidium neolycopersici*)

PM is an unambiguous biotrophic plant pathogen feeding on living tissue. Plant resistance to PM is connected to post-inoculation cell death and activation of the SA pathway (Thaler *et al.,* 2004), while jasmonate had no impact on fungi development (Thaler *et al.,* 2004). An effective plant defence against biotrophic fungus is mainly dependent on SA (Wang *et al.,* 2011) and during PM pathogenesis, fungi tries to suppress a host cell death (Hückelhoven *et al.,* 2011).

During PM infestation MT emissions were increased. Compared to GM infection, MT increases were small. The reason for this difference is the different fungus behaviour. GM, as a necrotroph, can destroy plant tissue including trichome damage in a relatively short time. Contrary, PM keeps cells alive (Hayes *et al.,* 2010) and therefore trichome damage of PM infected plants should be much lower. Although it is unlikely that PM grows on dead tissue such as trichromes, trichome damage may be caused by leaf senescence and wilting due to fungus parasitism. However, from the differences in life style of both pathogens, the differences in increases of MT emissions are understandable.

GLV emissions were not detected from PM infected plants. Martin *et al.* (2005) proposed that PM uses structurally and compositionally modified cell microdomains to penetrate the cell. PM uses only haustoria to take up cell nutrients, creating very little damage to the cell membrane (Hückelhoven *et al.,* 2011). The lack of GLV emissions is therefore explainable by the PM penetration strategy that just did not result in membrane damage severe enough to be detectable by GLV emissions.

Presence and/or increased emissions of induced compounds such as α-copaene result from the activation of signalling pathways in response to the biotic stress. Nevertheless, the data shown here do not allow any conclusions about activation of such pathways by PM. Induction of α-copaene emissions by PM infection was low, but the differences in α-copaene emissions

between PM infected plants and controls were still significant. In experiments with MeJA exposed plants (Chapter 3), α-copaene emissions have been induced, together with HexD and (*E*)-β-ocimene emissions, by activating the JA pathway. Furthermore, lack of HexD, (*E*)-β-ocimene and also GLV emissions indicates that JA pathway in PM infected plants was not strongly activated. This shows that, besides JA pathway, α-copaene can be induced also by other signalling pathways. Therefore, α-copaene emissions from PM infected plants cannot be associated with the activation of the SA or JA signalling pathways. During the experiments with PM infection of tomato, neither the SA nor the JA pathway was activated strongly enough in order to induce emissions that can directly be related to any of these pathways.

The data presented here are not in agreement with those of Quaglia *et al*. (2012) who report emissions of MeSA and MeJA from *Nicotiana tabacum* in response to attack by *Golovinomyces cichoracearum.* None of both emissions was detected here in response to PM. Reason for this could be just different plant and pathogen species. Qualia *et al.* (2012) and Ellis *et al.* (2002) show that in PM infected plants both, SA and JA pathways are active. However, just by observing increased SQT emissions from PM infected plants, it is impossible to confirm these findings.

Comparison of induced BVOC emissions between PM and GM infected plants, shows plants' different reaction to different fungi. From the expected behaviour of GM, from the visual symptoms, the temporal behaviour of MeSA emissions, and finally from the results of MeJA tests, I conclude that GM infected plants had predominantly the JA pathway activated. For PM infected plants, no reliable conclusions can be drawn with respect to the plants defence mechanisms. Reason therefore is a lack in induced emissions. A possible explanation for the lack of induced emissions is due to the less destructive PM infection at the stage where the plants were investigated.

5.4.3 Aphid (*Myzus persicae*)

The high release of MT from aphid-infested plants was most likely due to insect presence and movement on the surface of tomato leaves. The reproduction of aphids on tomato was not very fast (compared to kale) and the number of aphids remained relatively low. Correspondingly, MT emissions after six weeks aphid exposure were not significantly higher than after two weeks aphid exposure.

The effect of aphids on tomato plants seemed to be moderate. Visual symptoms were sparse even after 4 weeks of aphid exposure. I therefore assume that stress intensity was low. Since aphids did not reproduce very fast, I furthermore assume that tomato plants exhibit higher resistance to aphid attack than kale where aphids reproduced much faster. This higher resistance might be a reflection of the presence of tomato trichomes, which plays an important role in plant defence against aphids (Kang *et al.,* 2010; Walling, 2008).

Aphid feeding strategy of sucking nutrients from plant phloem doesn't cause extended membrane damage (Giordanengo *et al.,* 2010) and furthermore, a relatively small number (less than 100 per leaf) of aphid individuals was feeding on the plant. Thus, the absence of GLV emissions during aphid attack was not surprizing.

MeSA has been reported as a compound involved in indirect plant defence against herbivores (Dicke *et al.,* 1990). MeSA can attract their natural enemies (Zhu and Park, 2005) and it is repellent for aphids (Glinwood *et al.,* 2000). The finding of MeSA emissions being induced by aphid infestation is in agreement with the observations of Blande *et al.* (2010) and Zhu and Park (2005). However, it seems that the stress intensity during additional two weeks of aphid exposure was not increased enough in order to severely effect MeSA emission rates (Niinemets, 2010a), most likely due to relatively low increase in number of aphid individuals.

Ament *et al*. (2006) and Tholl *et al.* (2011) reported multiple step regulation of TMTT synthesis. Since TMTT was induced in both, control and aphid infested plants, the strong increase in TMTT emissions during aphid attack indicates the existences of multiple factors that are influencing TMTT emissions. TMTT is a compound that has been reported to be involved in the plants indirect defence mechanisms by attracting predators of herbivores (Kant *et al.,* 2004); therefore, increased TMTT emissions from aphid-infested plants are expectable.

During my experiments with aphids, *(E)*-β-ocimene emissions were not detected. Since low rates of aphid reproduction and lack of visual symptoms indicate mild stress, it is possible that *(E)*-β-ocimene emissions were too low to be detected. Furthermore, MeJA exposure experiments have shown that *(E)*-β-ocimene is induced by MeJA treatment, therefore lack of these MeJA induced emissions might indicate insufficient activation or a suppression of the JA pathway in aphid infested tomato plants. Despite of the low infestation, plants demonstrated a typical reaction to aphid infestation by emitting MeSA.

5.4.4 Whitefly (*Trialeurodes vaporariorum*)

Increases in constitutive emissions (MT and β-caryophyllene) from WF infected plants are expected due to presence and movement of insects on the plant surface. Although 80 % increase in MT emissions seems to be quite high, the effect induced by WF is relatively low compared to aphid infestation. The substantial difference in MT emission rates between aphid and WF exposed plants could be due to differences in insect behaviour or plant growing environmental conditions (see Chapter 2) that might affect trichome density (Wilkens *et al.,* 1996). Although WF feeding on plant causes only minimal membrane damage (Walling, 2008), some GLV emissions, predominantly (*Z*)-3-hexenol, were detected from WF infested plants. Lower amounts of (*Z*)-3-hexenol, though, were emitted from control plants, too. Since GLV could not be detected from WF plants by using JPAC GC-MS system, that can be calibrated, only an upper limit for these emissions can be estimated. Using the detection limit of the JPAC GC-MS system (~ 1 ppt), the airflow used during these measurements (~5-7 L/min) and the leaf area of the investigated plants (150-220 cm²), (*Z*)-3-hexenol emissions must have been lower than $1 \cdot 10^{-16}$ mol·m^{-2}·s^{-1}. Comparing this value to the lowest detected (*Z*)-3-hexenol emission from ozone exposed plants ($7 \cdot 10^{-14}$ mol·m^{-2}·s^{-1}, Table 2), GLV emissions from WF infested plants are negligibly low, although still higher than those of control plants.

Just like in aphid-infested plants, several different factors might simultaneously influence TMTT emissions from WF infested plants. However, increase of TMTT emissions from WF-infested plants is expectable since TMTT plays a part of plants defence against herbivores (Kant *et al.,* 2004.).

MeSA was detected from WF infested as well as from control plants, but its emissions from WF infested plants were significantly higher. This is partially consistent with results from Ángeles López *et al*. (2012), who found MeSA emissions only in WF infested plants. Since absolute emission rates were not given by Ángeles López *et al. (*2012), it is impossible to predict how relevant MeSA emissions in their work are.

As no MeSA emissions could be detected by using JPAC GC-MS system whose detection limit for MeSA is $1 \cdot 10^{-16}$ mol·m^{-2}·s^{-1} (~1 ppt), it can be assumed that MeSA emission rates of WF infected plants must have been below this level. In control plants, MeSA emission rates were in average 10 times lower and thus negligible. Furthermore, both, WF infected and control plants emitted benzoic acid as well, which is synthesised *via* the SA synthesis pathway (Lee *et al.,* 1995). The presence of MeSA and benzoic acid emissions indicate activation of the SA pathway.

Except for TMTT, other stress-induced emissions found in control plants such as (*E*)-β-ocimene, MeSA and benzoic acid show that control plants were also exposed some level of unknown stress. However, when compared to WF exposed plants, these stress-induced emissions from control plants seem to be almost negligibly low, what indicates that stress in control plants was also extremely low.

5.5 Summary and conclusions

In this study, changes of BVOC emissions from tomato in response to different biotic stressors in a mild or an early stage were investigated. The BVOC emission patterns and emission strengths were different for the different stressors. Generally, the changes of emissions were explainable by the character of the stressor and its impact on plant.

This study shows that stress induced compounds such as MeSA, α-copaene, *(E)*-β-ocimene and HeXD can be used as indicators of biotic stress in tomato plants. Except for these compounds, biotic stress can also induce some stress unspecific compounds, such as GLV, or increase constitutive emissions such as MT and SQT. Furthermore, compounds that are known to be a part of specific signalling pathway, such as MeSA or benzoic acid, can provide additional information about plant reactions to stress.

Results from this study show that even mild or early biotic stress can induce BVOC emissions. However, despite obvious visual symptoms some biotic stresses such as powdery mildew can induce only minor changes in BVOC emissions. On the other hand, in aphid-infested plants, stress induced BVOC emissions were detectable even before obvious visual symptoms of injury. It seems that the more intensive and/or the more destructive the stress is, the higher are the induced emissions. Therefore, it is possible that necrotrophs can cause more obvious changes in BVOC emissions in a very early stage of stress than biotrophs. Results from this study indicate that changes in BVOC emissions in a mild or early stage of biotic stress might be sufficient for stress detection or phenotyping.

6 TARGET BVOC EMISSIONS WITH POTENTIAL FOR DETECTING BIOTIC STRESS IN TOMATO GREENHOUSES

6.1 Introduction

Tomato production in large-scale greenhouses is characterised by monoculture and high plant densities throughout the year. This creates ideal conditions for the development and spreading of pathogen infections in greenhouses (van Lenteren, 2000). In order to keep yield loss caused by pathogens under control, tomato farmers depend on a preventive application of chemicals. In accordance with new environmental protection laws, farmers will have to reduce the amount of pesticides used in greenhouses.

There are several alternative methods for preventing pathogen infections in greenhouses. However, alternative methods for pathogen control are limited on such a great scale after infection has already occurred (personal communication with Dr. Jantineke Hofland-Zijlstra). Therefore, reduction in pesticide usage might result in severe yield losses due to pathogen attack. One reliable strategy to reduce yield loss due to pathogens is early stress detection. This allows an early management and application of pathogen control measures in stages when damage caused by pathogen is still relatively low.

Using BVOC emissions for detecting changes in different production systems is discussed since over a decade. Application of VOC sensors is already common in different areas such as oil industry, medicine or food production and storage (e.g. Patel *et al.,* 2003; Machado *et al.,* 2005; Mayr *et al.,* 2003). Also in greenhouse tomato production BVOC emissions should have a potential for early biotic stress detection. In the previous chapter, it was shown that biotic stress can induce new BVOC emissions which are normally not present in unstressed plants (controls). Some of these compounds can be used as biotic stress indicators. Detecting biotic stress induced emissions might help farmers to locate plants under early biotic stress in greenhouses before infection spreads on neighbouring plants.

This part of my study focuses on identifying target BVOC compounds that can be used for developing system for early biotic stress detection in a tomato greenhouse. Emissions from target compounds should be specific for plants under biotic stress, detectable at a very early stage of stress and the

emissions should be detectable at a greenhouse scale. I thus compared all BVOC emissions induced by biotic stress (caused by grey mould, powdery mildew, aphid and whitefly) to BVOC emissions induced by any other stress or event commonly occurring under greenhouse conditions. I termed these events greenhouse scenarios. Gas emissions from five different greenhouse scenarios, which might interfere with biotic stress detection were tested under laboratory conditions: mechanical injury, detached leaves, flowers, ripe fruits, and crushed tomato fruits. Furthermore, I will mention some possibilities for application of target compounds for biotic stress detection in greenhouses.

6.2 Specific materials and methods

6.2.1 Detached leaves, flowers and fruits

Detached leaves, fully developed flowers, and red fruits were collected from several living tomato plants and placed in the measuring chamber in a random order right after detachment. For calculating emission rates, detached flowers were weighed after exposure to dry air for 24 hours. Emissions from fruits were first measured from undamaged fruits for several hours, and then the same fruits were used for measuring emissions after crushing them. Fruits were crushed inside of the measuring chamber by using a mortar and pistil. After this process, BVOC measurements started right away and lasted for about 10 hours. Every experiment was performed six times.

6.2.2 Mechanical injury

A tomato plant was first introduced into the measuring chamber. After constitutive emissions stabilised, the plant was handled roughly, which led to severe mechanical injury. As rough handling and detachment of leaves resulted in the same emissions, the experiment with mechanically injured plants was repeated only three times.

6.3 Results

GLV were detected in all greenhouse scenario experiments except for undamaged fruits.

In experiments with mechanical injury and detached leaves, only constitutive MT, SQT and GLV were detected (Table 15). Emission rates of GLV were highly variable between individual treatments and their repetitions. Detected GLV were 2-penten-1-ol, (*E*,*E*)-2,4-hexadienal, (*E*)-2-hexenal, (*Z*)-3-hexenal, hexanal and (*Z*)-3-hexenol as the strongest emission (Table 15). Emissions of all emitted GLV were correlated (R^2>0.9) (Figure 21) and, therefore (*Z*)-3-hexenol was used as a representative of all GLV. In mechanical injury and detached leaves tests, bursts of GLV were followed by an increase in MT emissions. In detached leaves and after mechanical injury of the whole plant, MT and GLV emission decreased back to amounts similar to those of control plants. However, the time needed for GLV emissions to decrease reflected the severity of injury. For example, GLV from detached leaves dropped to below the detection limit within 2 hours, while in crushed plants, GLV were detectable for 3-4 hours after injury.

Constitutive MT emissions were present in all experiments regardless of treatment. After mechanical damage the release of constitutive MT increased and these increases seemed to be related to the severity of the injury. The emission pattern of the constitutive MT was constant and did not change with and without mechanical damage i.e. the releases were strongly correlated (R^2 >0.9). Release of β-phellandrene was the highest, identical to the constitutive tomato emissions from undamaged plants. SQT, such as β-caryophyllene and δ-elemene were too low to be detectable in all chromatograms and therefore not reported here (Table 15).

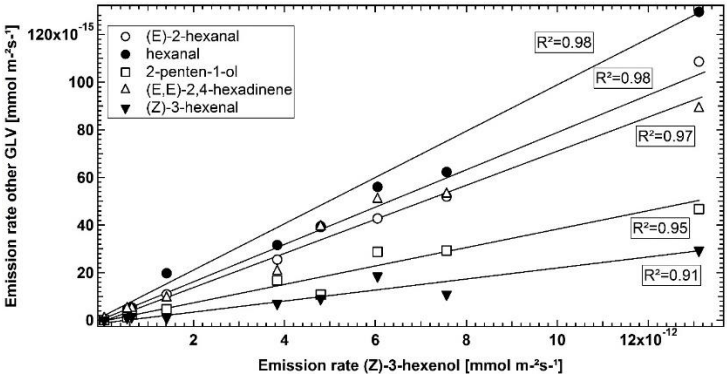


Figure 21 - Plot of GLV emissions (2-penten-1-ol, (*E,E*)-2,4-hexadienal, (*E*)-2-hexenal, (*Z*)-3-hexenal, hexanal) from tomato plants after mechanical injury as function of (*Z*)-3-hexenol emissions.

Table 15 - List of detected compounds from "greenhouse scenarios" experiments and their average values for the highest emission rates; MeSA - methyl salicylate, - - below detection limit and below $1 \cdot 10^{-16}$ mol·m^{-2}·s^{-1}, DW - dry weight, FW - fresh weight

TREATMENT	(*Z*)-3-hexenol	α-terpinene	(*E*)-β-ocimene	MeSA
Detached leaves mol·m^{-2}·s^{-1} (six tests)	$(9.7 \pm 3.4) \cdot 10^{-12}$	$(3.8 \pm 1.3) \cdot 10^{-13}$	-	-
Mechanical injury mol·m^{-2}·s^{-1} (four tests)	$(4.5 \pm 1.2) \cdot 10^{-11}$	$(6.7 \pm 4.5) \cdot 10^{-12}$	-	-
Detached flowers mol·g^{-1} DW·s^{-1} (~ 15 flowers) (six tests)	$(3.3 \pm 1.4) \cdot 10^{-14}$	$(7.8 \pm 3.5) \cdot 10^{-14}$	$(3.0 \pm 1.3) \cdot 10^{-14}$	$(8.2 \pm 3.6) \cdot 10^{-16}$
Undamaged ripe fruits mol·g^{-1} FW·s^{-1} (six tests)	-	$(3.5 \pm 0.2) \cdot 10^{-8}$	-	$(1.3 \pm 0.5) \cdot 10^{-6}$
Smashed ripe fruits mol·g^{-1} FW·s^{-1} (six tests)	$(6.5 \pm 2.5) \cdot 10^{-5}$	$(1.1 \pm 0.4) \cdot 10^{-7}$	-	$(1.7 \pm 0.7) \cdot 10^{-6}$

TMTT emissions were detected from mechanically injured plants and flowers, but not from tomato fruits or detached leaves. In experiments with whole plants, mechanical injury had no impact on TMTT emission rates.

α-copaene and HexD emissions were not detected under any of the tested greenhouse scenarios; however, MeSA emissions were detected from flowers and tomato fruits (Table 15). Besides TMTT and MeSA, detached flowers also emitted *(E)*-β-ocimene (Table 15).

6.4 Discussion

Study of biotic stress (Chapter 5) showed that biotic stress in tomato plants can induce GLV, HexD, *(E)*-β-ocimene, α-copaene and MeSA emissions. These stress-induced BVOC were considered as compounds with potential for biotic stress detection in greenhouses. Each compound and the advantages or disadvantages for biotic stress detection will be further discussed for selecting target compounds.

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

Lack of TMTT emissions in detached leaves can be explained by the fact that detached leaves, after entering the measuring chamber, have dried out within one to two hours. This time period is too short to induce TMTT emissions (compare to Figure 11). In all previously tested living plants, TMTT was induced after plant has spent several hours in a measuring chamber.

TMTT emissions were observed from all tested living plants (treatments and controls). I therefore cannot recommend using TMTT emissions as indicator of biotic stress. As long as there is no detailed information on the elicitor of the emissions and the emission behaviour in real greenhouses, TMTT is not considered as a target compound.

6.4.2 Constitutive monoterpenes and sesquiterpenes

Additional releases of constitutive MT and SQT are not specific to a certain stress. Simple plant movement or touching of the plant surface may cause trichome breakage and increase of constitutive emissions. Increased constitutive MT and SQT emissions alone do not specifically indicate biotic stress and they appear in all greenhouse scenarios. Increases of constitutive MT and SQT emissions are not applicable for early biotic stress detection.

6.4.3 Green leaf volatiles

Strong bursts of GLV emissions as also detected in almost all greenhouse scenarios indicate membrane damage (Croft *et al.,* 1990). Mechanical injury and membrane damage during routine greenhouse work is common. Hence, bursts of GLV emissions are also unspecific for stress.

However, Jansen *et al.* (2009c) have shown that GLV were not present in the greenhouse before shoot removal or fruit picking. On the greenhouse scale, GLV emissions increased due to mechanical injury supporting the hypothesis that GLV are not typically present in greenhouse air. GLV emissions are induced by workers activity. Under intensive greenhouse production, the number of workers who are in direct contact with plants is rather limited with exception of trellising, shoot removal and harvest (personal communication with Dr. Roland Mumm), which reduces the frequency of accidental membrane damage caused by labour. Furthermore, almost all management measures in greenhouses are tightly controlled. Thus, GLV emissions in a greenhouse without intensive workers activity are most likely associated to biotic stress.

The advantage of GLV as a target compounds are the high emission rates (e.g. Table 10 – the highest of all tested stresses). GLV are emitted within minutes after membrane damage (Loreto *et al.,* 2006). Additionally, GLV emissions have been reported from tomato plants infected with other biotic stresses caused by tobacco hornworm (*Manduca sexta*) (Farag and Paré, 2002; Degenhardt *et al.,* 2010) and oriental leafworm moth (*Spodoptera litura*) (Raghava *et al.,* 2010).

I conclude that GLV are usable as indicator of stress in greenhouses when routine work is halted. However, the best way to use GLV as a target compounds is in combination with other target compounds.

6.4.4 (*E*)-β-ocimene

Early or mild biotic stress induced only low (*E*)-β-ocimene emission rates (see Chapter 5). As an example, (*E*)-β-ocimene emissions from WF infected plants were only detectable after 24h gas sampling. Detection on a greenhouse scale therefore is either too insensitive or requires too much sampling time, what makes early stress detection very limited. Therefore, according to results from this study, (*E*)-β-ocimene is most likely not a good target compound for biotic stress detection in a greenhouses.

6.4.5 α-copaene

α-copaene emissions emitted from plants infested by WF, PM and GM infections were much higher than *(E)*-β-ocimene emissions and by far higher than emissions from control plants. Emissions of α-copaene were not found in greenhouse scenario experiments, suggesting that α-copaene is a suitable candidate target compound for stress detection in tomato greenhouses.

Besides α-copaene, Moneymaker emits other SQT such as β-caryophyllene, α-humulene and δ-elemene (Schilmiller *et al.,* 2010). My results show that emissions of constitutive SQT are very low and often below the detection limit of the here used analytical devices. However, emissions of constitutive SQT still can be increased by trichome damage (Jansen *et al.,* 2009c). In certain sensors, such as biosensors or electrochemical sensors, the principle of detection is based on chemical reaction of target compound and surface of the sensor. Therefore, these sensors are designed to detect only specific compound or group of compounds with similar molecular shape. In this kind of sensors, presence of other SQT might interfere with α-copaene detection (personal communication with Dr. Ramaraja Ramasamy). In this case, the possible advantage could be that α-copaene is the only tricyclic SQT detected from Moneymaker, what might play a crucial role in designing electrochemical sensor or biosensor for α-copaene detection in the greenhouses.

6.4.6 Methyl salicylate

High MeSA emissions have been found in tomato plants caused by phloem-feeding arthropods (see Tables 13 and 14), including spidermites (*Tetranychus urticae) (*Kant *et al.,* 2004). However, MeSA is also emitted from flowers and ripe fruits (Table 15). Hence, MeSA may be found in a greenhouse although there is no biotic stress. The use of MeSA emissions as a target compound might be limited predominantly by MeSA emissions from ripe tomato fruits. However, MeSA emissions due to biotic stress were quite high and common. Hence, MeSA emissions might be usable as a target compound. Its application is likely to be limited to periods without ripe fruits or only in some parts of the plants. For example, in Dutch type of greenhouses, tomato leaves that grow under the oldest truss are removed. That way, tomato fruits and flowers are directly exposed in a lower part of plant while tomato leaves remain on the upper part of the plant (personal communication with Jean-Marie Michielsen). The main GM infection sites in tomato greenhouses are injured stems rather than leaves (personal communication with Dr. Jantineke Hofland-Zijlstra). Therefore, biotic stress detection by MeSA emissions should be focused predominantly on leaves, as an early warning system for phloem-feeding pests rather than stems and GM infections. Due to vertical movement of the air, this kind of approach is not possible by sampling the air from several plants simultaneously. However, very small sensors such as biosensors or electrochemical sensors, that can be placed on each individual plant, and therefore, close to the infection spots, might be able to detect gas emissions emitted locally from only one part of the plant. Whether tomato fruits emit MeSA also during the ripening process and whether it is possible to detect MeSA originating from infested leaves only and not tomato fruits, still needs to be tested.

6.4.7 Hexenyl derivatives

HexD emissions in biotic stress tests were found only in GM infected plants and no emissions have been found in any of the tests related to greenhouse scenarios. Reports show that HexD can also be induced by herbivore feeding (Raghava *et al.,* 2010; Degenhardt *et al.,* 2010).

Just as in the case of α-copaene, the disadvantage of HexD compounds as target compounds for biotic stress detection in greenhouses might be in their similarity to chemical structure of GLV (Umasankar *et al.,* 2012). By using sensors, such as biosensors or electrochemical sensors, that

are designed to detect only one group of compounds, GLV might be falsely detected as a HexD.

6.4.8 Detection of target compounds on a greenhouse scale

The stability of BVOC compounds in an environment depends on the concentrations of oxidants such as ozone, OH and NO_3 radicals in the gas phase of a greenhouse (Holopainen and Blande, 2013). However, ozone, that can enter a greenhouse trough the vents, is efficiently taken up by plants (Fares *et al.,* 2008). Dense plant covers in greenhouses cause a strong sink for ozone yielding very low ozone concentrations in greenhouses. Low ozone concentrations cause low concentrations of the other oxidants such as OH and NO_3 radicals. The latter oxidants have atmospheric lifetimes in the range of seconds. Hence, concentrations of radicals in the greenhouse air due to inflow by ventilation are negligibly low. The production of the latter radicals requires ozone to be present. Hence, at low ozone concentrations the concentrations of the other radicals are too low to cause important losses of BVOC. With respect to the possible reactions in the gas phase, the chosen target compounds should be stable enough to allow detection with the suited equipment. So far, previous reports show that α-copaene, MeSA and GLV can be detected in an air blend of a small greenhouse by using GC-MS and gas sampling for only one hour (Jansen *et al.,* 2009c).

In recent years, great progress has been made in the development of very sensitive and highly selective sensors for MeSA and GLV/HexD detection (Umasankar *et al.,* 2013; 2012). Biosensors and electrochemical sensors could be a cheap and fast option for stress detection on a greenhouse scale. These sensors have a great potential for application in agriculture and they can be easily designed for each target compound (personal communication with Prof. Spyros Kintzios). However, further greenhouse tests are necessary for choosing ideal sensor or sensor combinations for biotic stress detection by BVOC.

The results presented here show that, from the stress-induced compounds emitted by tomato plants, two compounds and two groups of BVOC can be used as target compounds for biotic stress detection in a greenhouse: α-copaene, MeSA, HexD and GLV.

6.5 Summary and conclusion

Results of this study show that not all biotic stress indicators can be used as a target compounds for biotic stress detection in the greenhouses. Such a biotic stress indicator is (*E*)-β-ocimene, whose emission rates are too low for mild stress detection. Some stress unspecific compounds, such as GLV can be used as target compounds only when workers activity in greenhouses is low. For biotic stress detection in greenhouses, two individual BVOC and two BVOC groups can be used as target compounds: MeSA, α-copaene, and HexD and GLV. Monitoring of all BVOC emissions from tomato plants is not necessary, as only detection of target compounds is required for biotic stress detection. Furthermore, it is not necessary to detect all target compounds. All four selected target compounds can indicate most common biotic stresses occurring under tomato production. The detection of just one or two of these target compounds can be sufficient to indicate biotic stress. Biotic stress detection by HexD and α-copaene, most likely, will be uncompromised by routine greenhouse work or different plant stages. Application of GLV and MeSA, on the other hand, might be limited to certain periods. During periods where biotic stresses may be mimicked by greenhouse scenarios GLV and MeSA can be excluded as target compounds to avoid false alarm.

7 GENERAL SUMMARY AND CONCLUSION

Plant BVOC play an important role in plant ecology. Their emissions are easily altered by different stresses. Two types of stresses play a particularly important role in tomato production – drought and biotic stress.

This thesis focuses on stress impact on tomato BVOC emissions. The main research objective on this study was to investigate if stress can cause changes in tomato BVOC emissions and whether these changes can indicate stress even at early stages.

Guided by the limitations in tomato production due to stress, two main research question complexes were formulated:

1. What are the impacts of drought on constitutive and induced BVOC emissions from tomato? Can BVOC emissions be used as an early indicator of drought?

2. What are the impacts of biotic stress on tomato BVOC emissions? Can BVOC emissions be used for early biotic stress indicators? If so, what are the target compounds for an early stress detection system in greenhouses?

The study of drought stress contemplated impacts of drought on two types of BVOC emissions in tomato plants – constitutive emissions and induced emissions. In order to investigate drought stress impact on induced BVOC emissions, emission rates of these compounds were first elevated to above the detection limit of the analytical device by application of high ozone concentrations or fumigation with MeJA. Ozone exposure in tomato plants induced MeSA, HexD and (*E*)-β-ocimene emissions, but the emission rates ceased within several hours. High ozone concentrations also lead to the development of necrotic spots and severely decreased plant transpiration. MeJA exposures induced emissions of α-copaene, (*E*)-β-ocimene and HexD and within the first days of MeJA exposure, plants showed no obvious visual symptoms due to MeJA application. Therefore, MeJA fumigation was chosen as a reliable method for inducing tomato BVOC emissions in the further study of drought impact on induced BVOC emissions in tomato plants.

Results from the drought study show that the impact of drought on tomato BVOC emissions depends on two major factors: type of emissions and

severity of stress. Some general responses were found. Severe drought resulted in trichome damage and increased release of the MT stored in the trichomes. Membrane damage and bursts of GLV emissions appeared at the very late stage of drought, close to the plant death. On the other hand, intermittent increases of *de-novo* emissions of TMTT, (*E*)-β-ocimene, and the HexD were observed from well watered plants as well as during mild drought. Such increases were not attributed to the impact of drought.

With increasing severity of drought, emissions of TMTT, (*E*)-β-ocimene and HexD decreased. Compared to the drought induced decreases of transpiration and net photosynthesis, the decreases in BVOC emissions were delayed. The delay can at least partially be explained by the use of alternative carbon sources for the biosynthesis of the respective BVOC, however, the delay itself makes detection of drought stress by BVOC emissions unfeasible. I found no BVOC changes that can be attributed specifically to drought.

Constitutive emissions in tomato plants play a major role in pest-host recognition (Kang *et al.,* 2010) while both, constitutive and induced emissions are an important part of plant defence and communication (Dicke, 2009; Arimura *et al.,* 2005; Pickett *et al.,* 2003). The here presented experiments with MeJA induced emissions show that even in plants with predominantly activated JA pathway, severe drought stress still suppresses induced emissions. Therefore, it is possible that drought can hamper the plant's ability to communicate and/or defend itself from other organisms. However, whether such changes will make any difference in yield loss of already severely drought stressed plants growing in the field, still needs further testing.

In tomato greenhouse production, major yield losses are caused by biotic stresses such as grey mould (*Botrytis cinerea*), powdery mildew (*Oidium neolycopersici*), aphid (*Myzus persicae*) and whitefly (*Trialeurodes vaporariorum)*. The study of early or mild biotic stresses has shown that changes in BVOC emissions are dependent on the stressor, type of damage, and severity of the stress. For example, severe membrane damage and strong bursts of GLV were found only in plants suffering from development of necrotic spots such as caused by *Botrytis cinerea*. Similar intensive bursts of GLV were not found with a pathogen that is causing only chlorosis and minimum membrane damage such as powdery mildew and phloem-feeding insects. Increases in MT emissions were observed in all stressed plants. Increases in the emission strengths can be attributed to insect movement, leaf wilting or necrosis and differences in the increases reflect the amount of trichome damage.

Results from my biotic stress study show that different sources of biotic stress can induce different *de-novo* emissions in tomato plants. Some *de-novo* emissions can be directly attributed to the activation of specific signalling pathways as a response to biotic stress. Such emissions are MeSA emissions associated with the activation of the SA pathway. Furthermore, emissions induced after MeJA exposure and JA pathway activation can also be induced by biotic stresses. Such emissions are α-copaene, (*E*)-β-ocimene and the HexD. *De-novo* induced compounds reflect the plants reaction to the presence of pathogens or parasites, and they can directly indicate biotic stress. My results show that the best biotic stress indicators for tomato are MeSA, α-copaene, (*E*)-β-ocimene and HexD (with exception of TMTT). Whereas monitoring of a single compound cannot indicate the nature of the biotic stress, measurements of all relevant emitted compounds together might yield more information about the nature of the stress.

Induced BVOC emissions together with visual symptoms might give an insight on the underlying plant responses to the necrotrophic pathogen *Botrytis cinerea* and phloem-feeding insects such as *Myzus persicae* and *Trialeurodes vaporariorum.* These findings show that BVOC might have a potential in plant phenotyping.

MeJA induced BVOC emissions have a potential for providing information about active JA pathway. Further tests should include inducing BVOC emissions by activation of the SA pathway only. That could provide a tool for identifying BVOC emissions that are directly associated with active signalling pathway. This kind of study should be completed by metabolic analysis such as RNA extraction and analysis of JA-dependent gene expression *PI* and *PII*, and SA and JA quantifications (for more details see El Oirdi *et al.,* 2011). Further studies on plant biotic stress should include tests of BVOC emissions from tomato cultivars with different resistance levels to specific biotic stressors. This kind of study could provide information whether resistant genotypes can be discerned from susceptible genotypes simply by observing changes in BVOC emissions.

Conclusions from this study are that BVOC emissions can be indicators of biotic stress at very early stages. For stress detection in tomato greenhouses, no complete gas monitoring is needed; instead, detection of four target compounds is sufficient. Biotic stress detection by two target compounds, α-copaene and any of the HexD, most likely will not be compromised by routine greenhouse work or plant stage. These two compounds should be sufficient for detection of *Botrytis cinerea* infections at very early stages. However, two other target compounds, MeSA and any of the GLV, have a great potential in stress detection in greenhouses but their

application may be limited. MeSA emissions showed to be good indicators of early infestations of *Myzus persicae.* But, as MeSA is also emitted from red tomato fruits, the presence of ripe fruits has to be taken into account. Similar to MeSA, GLV in greenhouses can be used as a general stress indicator. However, use of GLV as target compounds is possible only in situations when an accidental mechanical injury is excluded, such as during the night or when workers are not in direct contact with the plants.

The results from this study suggest that early biotic stress detection in greenhouses is possible, but future studies should follow. For example, Moneymaker is a very well investigated tomato cultivar, but it is not a cultivar common in commercial production on a larger scale (personal communication Prof. Heiner Goldbach). Therefore, further studies should include testing different commercially important tomato cultivars. Furthermore, it should be tested if pesticide application or a mild salt stress (used to improve the fruit taste) might interfere with biotic stress detection. Final application tests must be conducted under conditions of commercial greenhouse production.

At the end, this study provides a scientific base and further encouragement for developing BVOC detection system that can be directly applied in tomato breeding and production. This kind of detection system might play an important role in creating more sustainable greenhouses without simultaneously risking yield loss.

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