

Volatile emissions of *Puccinia triticina* infected wheat and its effect on uninfected wheat seedlings

by

Howard Dean Castelyn

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Supervisor:

Dr B Visser

Department of Plant Sciences

UFS

Co-supervisor:

Prof ZA Pretorius

Department of Plant Sciences

UFS

“Whenever you find yourself on the side of the majority, it is time to pause and reflect.”

Mark Twain

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Declaration

I hereby declare that this dissertation submitted for the degree of *Magister Scientiae* in Botany at the University of the Free State is entirely my own independent work. This dissertation has not previously been submitted by me at any other higher education institution. I furthermore cede copyright of this dissertation to the University of the Free State.

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Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
AOS	Allene oxide synthase
<i>Avr</i> gene	Avirulence gene
CR	Control resistant
CS	Control susceptible
DMAPP	Dimethylallyldiphosphate
DMDC	Dimethyldicarbonate
dNTP	Deoxyribonucleotide triphosphate
dpi	Days post infection
EDTA	Ethylenediaminetetraacetic acid
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
GC/MS	Gas chromatography mass spectrometry
GLV	Green leaf volatile
H ₂ O ₂	Hydrogen peroxide
HIPV	Herbivore-induced plant volatile
hpe	Hours post exposure
HPL	Fatty acid hydroperoxidelyase
HR	Hypersensitive reaction
IPP	Isopentenylidiphosphate
IR	Infected resistant
IS	Infected susceptible
LIR	Later infected resistant
LIS	Later infected susceptible
LOX	Linoleate oxygen oxidoreductase
MOPS	3-(N-Morpholino)propanesulfonic acid
MR	Mock infected resistant
MS	Mock infected susceptible
O ₂ ⁻	Superoxide anion
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular pattern

PMSF	Phenylmethanesulfonyl fluoride
PR	Pathogen-related
PRR	PAMP recognition receptor
PTI	PAMP triggered immunity
<i>R</i> gene	Resistance gene
RT-PCR	Reverse transcriptase polymerase chain reaction
SAR	Systemic acquired resistance
SPME	Solid phase micro-extraction
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloric acid
UR	Uninfected resistant
US	Uninfected susceptible

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Chapter 1: Introduction

Plants emit a vast array of volatiles into the surrounding atmosphere of which the composition differs between plant species, during the various life stages of an individual and in response to external factors. Research has shown that volatiles are essential in various interactions that occur between the plant and the environment (Durdareva *et al.*, 2006). One function of plant volatiles is to mediate interplant signalling as described in an early report by Baldwin and Schultz (1983). The theory maintains that volatiles released during infestation/infection may diffuse via the atmosphere to neighbouring plants to induce a defence response in these plants. This induced defence response in the exposed plants may then grant a competitive advantage during a subsequent challenge.

Volatiles are now firmly established as naturally emitted compounds that may cause plant defence response against pests and pathogens (Frost *et al.*, 2008; Haggag and Abd-El-Kareem, 2009). Research should further focus on interplant signalling to identify likely volatiles that act as signals, especially in food crops. These volatiles may prove to be advantageous in improving food security by a more environmentally sound method.

A signalling event was observed by Appelgryn (2007) in wheat (*Triticum aestivum*) infected with *Puccinia triticina* Erikss., the causal agent of leaf rust. In his study infected resistant (Thatcher+*Lr34*) and susceptible (Thatcher) wheat seedlings were independently placed in an enclosed chamber together with uninfected (both resistant and susceptible) wheat seedlings. An induction of expression of several defence associated genes was observed in the exposed uninfected seedlings, together with an increase in β -1,3-glucanase activity for certain combinations of Thatcher. The wheat seedlings had no contact except through the air in the enclosed chamber and signalling via released volatiles was thus the only plausible explanation.

The current study intends to verify and elaborate the research of Appelgryn (2007) by firstly addressing relevant criticism. One critique brought forward was that observing volatile effects in a small enclosed space is flawed, since this may allow compounds to accumulate to levels not present under field conditions (Dicke *et al.*, 2003; Paschold *et al.*, 2006). The experimental system of Appelgryn (2007) did not emulate natural conditions. Only of late were systems designed to allow for continuous air-flow between plants. These systems have been used to investigate various aspects of plant volatile signalling (Pettersson *et al.*, 1999; Ninkovic *et al.*, 2002; Ninkovic, 2003; Paschold *et al.*, 2006; Ton *et al.*, 2006). These systems closely simulate natural conditions where volatiles are released into the atmosphere

and the dispersal to neighbouring plants is dependent on external factors. Furthermore, the experimentation in interplant signalling must expose uninfected plants to a mixture of volatiles from a natural source and exclusive signalling via the air currents must be confirmed.

The aim of the current project was to confirm the putative signalling event between leaf rust infected wheat and uninfected wheat as observed by Appelgryn (2007) by using a continuous air-flow system. The hypothesis was that seedlings infected with *P. triticina* emit volatile compounds that may diffuse to uninfected wheat seedlings in which a defence response is then induced. This defence response was to be confirmed by observing the induction of a number of defence markers. The second aim was to investigate whether any specific susceptible/resistant interactions were present and ascertain if the compatible and incompatible interactions elicited the same response in exposed plants.

Released volatile signals are present in low concentrations in the atmosphere surrounding a plant because of its rapid diffusion. Methods such as solid phase micro-extraction allow for the capture of plant volatiles as they are emitted into the atmosphere. Gas chromatography mass spectrometry in turn has allowed scientists to accurately identify and quantify these emitted volatiles (Engelberth *et al.*, 2004; Wright *et al.*, 2005; Paschold *et al.*, 2006). This approach has been employed in the study of different interactions involving plant volatiles. Therefore the final aim of the current study is to identify volatiles emitted by the two Thatcher lines upon *P. triticina* infection in order to identify the putative volatile signal that may be responsible for the induced defence response.

Chapter 2: Literature review

In South Africa, 2.005 million tons of wheat (*Triticum aestivum* L.) was commercially produced during the 2011/2012 season but this was still less than the domestic requirement (SAGL, 2012). Local wheat production should increase, but the production of wheat (and indeed other crops) is challenged by a number of plant pests and pathogens. The worldwide crop yield loss due to various pests and diseases in wheat, rice, maize, barley, potatoes, soybean, sugar beet and cotton accumulated to 32% during the period of 1996-1998. Fungi and bacteria contributed 9.9% of total yield loss (Oerke and Dehne, 2004). In this constant struggle against plant fungal pathogens, new and creative methods must be found to reduce crop losses. Approaches that move away from the intensive use of fungicides are desirable as these chemicals may be detrimental to the environment. Cultivated land where copper fungicides were once used not only accumulated copper but the microbial diversity in the soil was lower than that of natural soil (Viti *et al.*, 2008). Alternatively science may look to induce the inherent plant defence response to battle pathogens by environmentally sound methods.

2.1 The plant defence response

Plant defence can be divided into preformed constitutive defence and an inducible defence response. The first constitutive defence barrier that is present in plants to prevent infection by pathogens is the cuticle of epidermal cells and suberized cell walls, that contain cutin and suberin respectively (Koiattukudy, 1985). Cutin and suberin are hydrophobic fatty acid-like polymers that resist biological degradation except by specialized enzymes. It should be noted that certain pathogens like *P. triticina* do not penetrate the epidermis directly but rather do so via the stomatal opening (Bolton *et al.*, 2008b).

Plant cells may also accumulate secondary metabolites that are directly detrimental to the pathogen with phytoalexins and saponins serving as examples. Various *Arabidopsis* mutants with defective phytoalexin synthesis (*pad* mutants) were more susceptible to *Peronospora parasitica* (Pers.) Fr. infection (Glazebrook *et al.*, 1997), while the *pad3* mutant in turn showed higher susceptibility to *Alternaria brassicicola* (Schwein.) Wiltshire. (Thomma *et al.*, 1999). Saponin deficient oat mutants (*sad* mutants) were also shown to be more susceptible to *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier. infection (Papadopoulou *et al.*, 1999).

The inducible defence response on the other hand is much more complex. Plant cells recognize pathogens that penetrate the cell wall through pathogen-associated molecular patterns (PAMP's) that are bound by PAMP recognition receptors (PRR's) which accordingly induce PAMP triggered immunity (PTI) (Schwessinger and Zipfel, 2008). PTI is complex and includes a number of induced molecular and physiological changes. PAMP's are molecules that originate from the pathogen and include flagellin, glucan, chitin and ergosterol (Nürnberg *et al.*, 2004). Plants have different PRR's that bind PAMP's thereby activating a signalling cascade. Rice receptor proteins that bind chitin were shown to be part of a signal cascade that induces defence responses such as phytoalexin accumulation (Ito *et al.*, 1997). The receptor protein was later identified and called chitin oligosaccharide elicitor-binding protein (Kaku *et al.*, 2006). In *Arabidopsis* a receptor-like protein kinase was shown to be responsive to chitin elicitation causing the downstream activation of mitogen-activated protein kinases, gene expression and the production of reactive oxygen species (Miya *et al.*, 2007). *Arabidopsis* mutants with a defective chitin responsive receptor-like kinase was only slightly more susceptible to *A. brassicicola* implying that additional PAMP's and signal cascades may contribute to PTI.

Plants are naturally resistant against the majority of pathogens as PTI successfully suppresses pathogen growth. However, pathogens have developed effector molecules that when secreted into the plant cell, suppress PTI leading to host specific basic compatibility (effector triggered susceptibility (ETS)). A differentiation should be made between biotrophic pathogens (growing on living plant tissue) and necrotrophic pathogens (growing on necrotic plant tissue) based on the functioning of the respective effectors (Johal *et al.*, 1994, Glazebrook, 2005). The effectors of biotrophic pathogens allow the fungus to remain undetected while still suppressing the host defence response (Johal *et al.*, 1994). *Septoria lycopersici* Speg. overcomes the constitutive defence response by the enzymatic activity of tomatinase that degrades the antifungal saponin α -tomatine (Bouarab *et al.*, 2002). The product of this degradation, β_2 -tomatine, was shown to act as an effector to suppress the hypersensitive reaction of *Nicotiana benthamiana* Domin. against both *S. lycopersici* and *Pseudomonas syringae* Van Hall.

Necrotrophic pathogens in turn form toxic compounds that act as effectors which interfere with the plant defence response (Johal *et al.*, 1994). One such toxin isolated from *A. brassicicola* spores was shown to mediate infection of various *Brassica* species (Otani *et al.*,

1998). When added to inoculum of non-pathogenic *Alternaria alternata* (Fr.) Keissl., the plant defence response was suppressed and infection proceeded on *Brassica* plants.

Effector molecules are normally products of pathogenic avirulence (*Avr*) genes that, when secreted into the plant cell, causes compatibility with the host plant. Plants in turn developed resistance genes (*R* genes) whose encoded polypeptides may recognize the products of the *Avr* genes (Jones and Dangl, 2006). Upon recognition and binding of the effector molecules, the plant initiates a strong active defence response (effector triggered immunity (ETI)) against the relevant pathogen. Plant-pathogen interactions are therefore referred to as gene-for-gene interactions since products of the *Avr* and *R* genes ultimately interact. Most *R* genes code for proteins that are involved in signal transduction associated with the recognition of the *Avr* gene product. Effector triggered immunity is usually associated with a hypersensitive reaction (HR) and numerous other defence mechanisms (Jones and Dangl, 2006).

The ETI defence response may include the so-called oxidative burst whereby reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) are formed (Wang *et al.*, 2010). H_2O_2 can crosslink glycoproteins in the cell wall and together with callose deposits reinforce the cell wall against further penetration (Brown *et al.*, 1998). The oxidative burst in most cases is associated with the induction of the HR. The HR is characterized by localized cell death in close proximity to the infection site of the pathogen and becomes visible as tissue necrosis on a plant. Such necrosis effectively restricts the spread of a fungal pathogen and in the case of biotrophs impedes the uptake of nutrients (reviewed in Mur *et al.*, 2008). The HR may also be observed in non-host interactions such as barley infected with *Blumeria graminis* (DC.) Speer. (Hückelhoven *et al.*, 2001) and tobacco infected with *P. syringe* (Keith *et al.*, 2003).

In a study by Bolton *et al.* (2008a), 151 differentially expressed genes were identified in *P. triticina* infected resistant wheat in comparison to uninfected resistant wheat. Upregulated genes included those coding for pathogen-related (PR) proteins, signal transduction components and other defence associated proteins. PR proteins are grouped into classes of which 17 are already recognized, even though the properties of some classes are still unknown (Van Loon *et al.*, 2006). Two classes of PR genes induced by pathogenic infection are PR2 (β -1,3-glucanases) and PR3 (chitinases). The activity of both these PR proteins is induced upon infection of wheat with *P. triticina* and may be correlated with resistance and appearance of the HR (Anguelova-Merhar *et al.*, 2001). β -1,3-glucanases hydrolyse the

β -1,3-bonds in glucan (Johal *et al.*, 1994), while chitinase in turn hydrolyses the β -1,4-bonds in chitin (Collinge *et al.*, 1993). Both glucan and chitin are complex polymers present in cell walls of pathogenic fungi. These PR proteins are therefore directly antifungal and effectively degrade the pathogen cell wall (Mauch *et al.*, 1988). The products of enzymatic PR proteins also act as elicitors of further defence response mechanisms (Fritig *et al.*, 1998).

The defence response is not only induced at the site of infection but also systemic in distal tissue. The response is known as systemic acquired resistance (SAR) and ensures that distal plant tissue acquires prolonged resistance against further pathogenic attack. This implies that a signal molecule is translocated from the local infection site to the systemic tissue (Sticher *et al.*, 1997). Numerous molecules have been implicated in SAR including salicylate, jasmonate, systemin and others (Sticher *et al.*, 1997, Vlot *et al.*, 2008). Methyl salicylate has been proven as the signal for SAR in tobacco (see also section 2.2.4.3) but this does not hold true for *Arabidopsis* (Attaran *et al.*, 2009). Jasmonate was implicated as the SAR signal in response to herbivory using grafting experiments (Li *et al.*, 2005). Lipid derived molecules have also been implicated as SAR elicitors. *Arabidopsis* mutants with defective lipid desaturase activity and lipid transfer protein could not induce SAR against *P. syringae* (Chaturvedi *et al.*, 2008). Loss of acyl-CoA oxidase activity (key enzyme in lipid metabolism) in infested tomato causes increased susceptibility to *Manduca sexta* L. worms and the inability to systemically induce proteinase inhibitors (Li *et al.*, 2005).

Finally, a primed defence response should be distinguished from a true induced defence response. An elicitor that primes a response increases the basal level of the defence response but only once a stress factor is recognized, is the typical defence response induced. A primed plant however has a stronger and quicker response than non-primed plants (Ahmad *et al.*, 2010). Ton *et al.* (2009) proposed a hypothesis whereby priming leads to the production of defence associated transcription factors. Once a stress factor is recognized, transcription can proceed and the response by extension is quicker as transcription factors need not be synthesized first.

The various molecules that play a role during the plant defence response include plant volatiles. These are molecules with a vapour pressure high enough to be emitted in the gaseous phase from plant tissues.

2.2 Classification and biosynthesis of volatiles

Volatile organic compounds (henceforth referred to as volatiles) are synthesized in various plant tissues. Biosynthesis may occur in epidermal cells for quick release into the atmosphere (Kolosova *et al.*, 2001a), in specialized secretory cells as seen in sweet basil (Gang *et al.*, 2001) or in glandular trichomes as with peppermint (McConkey *et al.*, 2000). The plastids and cytosol are the main cellular localities of volatile synthesis (Durdareva *et al.*, 2006). As these volatiles are secondary metabolites, the localization of synthesis is often determined by the biochemical pathway and intermediates involved. Such pathways are often extensive involving numerous enzymes and proceeds from one cellular compartment to another.

Literature often refers to artificial groupings of volatiles with representatives from different metabolic classes, such as the herbivore-induced plant volatiles (HIPV's) (Arimura *et al.*, 2009). Volatiles can however be arranged into only four true biochemical groups based on the source from which it is derived: terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives (Durdareva *et al.*, 2006).

2.2.1 Terpenoids

Terpenoids form the largest and most varied group of plant volatiles, and may also be referred to as terpenes or isoprenoids. These metabolites consist of five carbon isoprene as the basic subunit. Isoprene molecules are however not directly polymerized, but are derived from the same source as other terpenoids (Gershenson and Kries, 1999). Terpenoids are arranged in sub-groups with the classification based on the number of isoprene subunits present namely hemiterpenes, monoterpenes, sesquiterpenes, homoterpenes, diterpenes, tetraterpenes and polyterpenes (Durdareva *et al.*, 2006). Volatiles are found in a number of these sub-groups with isoprene, monoterpenes and sesquiterpenes (to a lesser extent) being predominant in the atmosphere (Kesselmeier and Staudt, 1999).

The synthesis of all terpenoids starts with isopentenylidiphosphate (IPP) that is derived from either the mevalonic pathway in the cytosol or the methyl-erythritol phosphate pathway in the plastids (Durdareva *et al.*, 2006). IPP readily isomerizes to dimethylallyldiphosphate (DMAPP), establishing a metabolic pool of both these isomers. Isoprene may then be produced directly from DMAPP by isoprene synthase (Gershenson and Kries, 1999). IPP in

the cytosol is a precursor for homoterpenes and sesquiterpenes amongst others, while IPP in the plastids is a precursor for isoprene, other monoterpenes and volatile carotenoids.

The enzymatic binding of IPP and DMAPP produces geranyldiphosphate, the precursor of monoterpenes (Gershenzon and Kries, 1999). The addition of another IPP molecule to geranyldiphosphate forms farnesyldiphosphate, the precursor of homo- and sesquiterpenes. The further addition of an IPP molecule to farnesyldiphosphate forms geranylgeranyldiphosphate, the precursor of diterpenes. Each successive polymerization step releases pyrophosphate, which undergoes hydrolysis to supply energy for the reaction. Further enzymatic reactions eventually lead to more complex terpenoids like tetraterpenes and polyterpenes (Gershenzon and Kries, 1999).

2.2.2 Phenylpropanoids and benzenoids

Phenylpropanoids and benzenoids are phenolic compounds (Petersen *et al.*, 1999). These two compounds are grouped together since both are derived from the amino acid phenylalanine. Phenylalanine is formed via the shikimic acid pathway in plastids, and transported to the cytosol for further catalysis. The first enzymatic reaction catalyzed by phenylalanine ammonia-lyase (PAL), is shared by phenylpropanoids and benzenoids. PAL converts phenylalanine to *trans*-cinnamic acid in the cytosol, where after the synthesis pathways of phenylpropanoids and benzenoids diverge (Durdareva *et al.*, 2006).

Gang *et al.* (2001) proposed a pathway whereby *trans*-cinnamic acid is first converted to coumaric acid, while phenylpropanoids like methyleugenol and methylchavicol are subsequently derived from this molecule (Boatright *et al.*, 2004). Other phenylpropanoids like phenylacetaldehyde (and further derivatives) may be synthesized directly from phenylalanine under the action of phenylacetaldehyde synthase (Boatright *et al.*, 2004; Durdareva *et al.*, 2006).

CoA-dependent β -oxidative and CoA-independent non- β -oxidative reactions are involved in producing benzenoids, even though many intermediates are shared between the two pathways (Petersen *et al.*, 1999). The CoA-independent non- β -oxidative reactions are probably of greater importance as the flux of certain intermediates through this pathway was proven to be twice as much as the alternative (Boatright *et al.*, 2004). A key enzyme in benzenoid

synthesis is *S*-adenosyl-L-methionine salicylic acid carboxyl methyltransferase, which catalyzes the production of methyl salicylate from salicylic acid. Methyl salicylate is a volatile that has been implicated in plant defence, particularly in the elicitation of SAR (Park *et al.*, 2007).

2.2.3 Fatty acid derivatives

All fatty acid volatiles are products of the lipoxygenase pathway where the first step is catalyzed by linoleate oxygen oxidoreductase (LOX) (Feussner and Wasternack, 2002). Linoleic acid, linolenic acid and arachidonic acid are substrates for LOX while the fatty acid hydroperoxide is produced. Variations of the LOX enzyme are present in many different cellular localities, all of which are organized in two sub-groups, LOX-9 and LOX-13. LOX-9 oxidizes the ninth carbon of the fatty acid chain, while LOX-13 the thirteenth carbon. The fatty acids required for the LOX pathway are synthesized in the cytosol from acetyl-CoA or are derived from the cellular membrane. The following steps in the LOX pathway occur in the cytosol and the products (oxylipins) have been implicated in plant defence (Prost *et al.*, 2005).

Fatty acid hydroperoxides can be converted by numerous enzymes, however only two are important for the eventual volatile production. Volatiles are derived from the products of either allene oxide synthase (AOS) or fatty acid hydroperoxidelyase (HPL) (Durdareva *et al.*, 2006). Products of AOS proceed via the so-called octadecanoid pathway which leads to the production of jasmonate and its derivatives. One of these, methyl jasmonate is synthesized by jasmonic acid carboxyl methyl transferase (Howe and Schilmiller, 2002). The addition of a methyl group to jasmonate not only increases volatility but also general translocation. Methyl jasmonate is transported more effectively in plant tissues via the xylem, phloem and across membranes compared to jasmonic acid (Thorpe *et al.*, 2007). Methyl jasmonate has been proven to directly induce numerous defence responses in plants (Seo *et al.*, 2001; Tschardt *et al.*, 2001; Jung *et al.*, 2007).

Green leaf volatiles (GLV's) are all derived from HPL products and are emitted by green foliage. Chemically GLV's are six carbon alcohols, aldehydes and corresponding esters, derived from six, nine or twelve carbon products of the lysis step catalyzed by HPL (Matsui, 2006). GLV's may accumulate in intact tissue but are otherwise synthesized rapidly upon

mechanical damage and have thus been implicated in numerous plant defence responses (Ruther and Kleier, 2005; Shiojiri *et al.*, 2006; Kishimoto *et al.*, 2008).

2.2.4 Amino acid derivatives

Except for phenylalanine, a number of other amino acids may also serve as precursors for volatile synthesis. Alanine, valine, methionine, leucine and isoleucine are all included (Durdareva *et al.*, 2006). When strawberry plants were grown in a medium supplied with additional isoleucine, an increase in fruit fragrance resulted (Pérez *et al.*, 2002). A total of 14 volatiles showed increases in concentration, with some volatile esters showing up to a seven-fold increase.

Amino acids are important for the production of nitrogen and sulphur containing volatiles, but may also act as precursors for molecules used in biosynthesis of other volatile classes. Amino acids can undergo deamination to produce the corresponding α -keto acids that are utilized in the synthesis of volatile esters and aldehydes (Durdareva *et al.*, 2006). Acetyl-CoA may also be derived from α -keto acids and is used in esterification by alcohol acetyltransferases, one example being the acetyl-CoA: geraniol/citronellolacetyltransferase in benzenoid synthesis (Shalit *et al.*, 2003).

One of the most important volatiles, ethylene, is derived from the amino acid methionine. Its biosynthesis proceeds from methionine to *S*-adenosylmethionine and then to 1-aminocyclopropane-1-carboxylic acid (ACC). Finally ACC is oxidized to form ethylene (Alexander and Grierson, 2002). The sulphur of methionine is recycled and used to synthesize another methionine by the Yang cycle (Yang and Hoffman, 1984). Ethylene fulfils various important functions and is the only volatile to have the status of plant hormone (Santner *et al.*, 2009).

2.3 Roles of volatiles in the environment

Volatiles may at first seem like a carbon loss to plants as these compounds are secondary metabolites that may be rapidly lost to the atmosphere. Some function must therefore be

attributed to these emissions to substantiate the carbon loss. Indeed many functions have been reported and will be discussed in greater detail in the following sections.

Halopainen (2004) proposed that volatiles function on four different levels, namely tissue, surface, ecosystem and atmospheric level. The concentration correspondingly decreases through these levels as volatiles diffuse away from the plant. At tissue and surface level volatiles are important in granting protection against abiotic stress factors, but at surface level also as direct defence against for instance pathogens. Volatiles at the ecosystem level are important in attracting pollinators and organisms in a tritropic interaction. Other plants may also perceive certain volatiles at ecosystem level and induce a subsequent response. At the atmospheric level, along with other gasses, plant volatiles play a role in various tropospheres.

2.3.1 Attraction of pollinators

The association of numerous volatiles with the reproductive structures of plants alludes to their role in mediating highly specific plant/pollinator interactions. In roses, the *RhAAT1* gene codes for an enzyme involved in volatile ester biosynthesis that is expressed solely in flowers (Shalit *et al.*, 2003). In snapdragon the enzyme responsible for methyl benzoate production is also exclusive to flower epidermal cells (Kolosova *et al.*, 2001a). Flowering plants may thus be expected to have a similar pattern of expression of key biosynthetic enzymes.

Kolosova *et al.* (2001b) measured the emission of methyl benzoate in relation to the dark/light cycle for snapdragon, tobacco and petunia plants. In snapdragon the release of methyl benzoate oscillated with a dark/light cycle, showing maximum emission during daytime. Since snapdragon plants are pollinated by bees, the emissions may be linked with the diurnal character of these insects. Tobacco and petunia plants in turn emitted maximum levels of methyl benzoate during the night, possibly pointing to nocturnal pollinators.

Honeybees can distinguish a group of snapdragon plants (with a high intensity of volatiles) from a single plant (with a lower intensity) as illustrated by Wright *et al.* (2005). The report also showed that honeybees can distinguish between different snapdragon cultivars, each with different ratios of volatiles (8 being measured and compared). The insects could not distinguish different individuals of the same cultivar, proving general homology in volatile

emissions for the given species. Therefore the perceivable concentration of volatiles and ratios of different volatiles allow pollinators to discriminate between plants.

Schiestl and Ayasse (2001) measured an increase in the emission of the terpenoid farnesylhexanoate after pollination of the orchid species *Ophrys sphegodes* Mill. Pollination occurs when male *Andrena nigroaenea* Kirby. bees mistakenly tries to copulate with what is perceived to be the female. Farnesylhexanoate is also released by female bees as a pheromone and lowers copulation attempts by males. The argument put forward is that increased levels of farnesylhexanoate might alter the behaviour of male bees in such a way to visit unpollinated flowers more often.

Negre *et al.* (2003) showed that after pollination and fertilization of snapdragon plants the level of all major floral volatiles decreased with time, irrespective of the time after anthesis. The decrease of methyl benzoate correlated with decreased gene expression and activity of the relevant synthetic enzyme. Thus once fertilization is successful no further pollinators are needed and the production of the related volatiles ceases. Muhlemann *et al.* (2006) measured the decrease of 28 volatiles after pollination of *Silene latifolia* Poir. and went on to argue that this is a mechanism to avoid seed predation. Volatile clues are usually being used by the seed predator *Hadena bicruris* Hufnagel. to locate the plant.

2.3.2 Development and fruit ripening

The most important volatile that induces various developmental changes in plants is ethylene. Underwood *et al.* (2005) observed that in petunia flowers the increased ethylene released after pollination regulates changes in the volatile profile and eventually causes senescence. Gene expression of synthesizing enzymes of methyl benzoate and methyl salicylate also decreased either upon pollination or ethylene treatment.

The role of ethylene in climacteric fruit ripening, in turn, is well established and has been exploited for many years by commercial fruit farmers. Ethylene is emitted once ripening starts and acts as volatile signal which induce further ripening, even in nearby fruits (Theologis, 1992). This volatile regulates gene expression, eventually causing changes in colour, softening of cell walls and increased ethylene production. While changes in the volatile profile make fruit more attractive, further ethylene release only hastens the ripening

process (Alexander and Grierson, 2002). Ethylene also induces fruit ripening in grapes, which are non-climacteric fruit that do not emit ethylene in large quantities. Specific stress conditions must however persist for this to occur (Tesniere *et al.*, 2004).

2.3.3 Volatile defence against abiotic stress

The emission of monoterpenes in *Quercus ilex* L. (Loreto *et al.*, 2004) and homoterpenes in lima beans (Vuorinen *et al.*, 2004) at high ozone levels may function as defence against oxidative damage. Ozone leads to the accumulation of reactive oxygen species in the cell, which in turn causes peroxidation of membrane lipids (Loreto and Velikova, 2001). Plants treated with fosmidomycin (an inhibitor of isoprene synthesis) showed increased ozone damage and decreased photosynthesis. Isoprene was speculated to stabilize the thylakoid membrane, lessening the damage by peroxidation and thereby protects the photosynthetic systems in the thylakoid membrane. Isoprene can also act directly to quench singlet oxygen, a reactive oxygen species that cannot be removed by any known enzymatic activity (Velikova *et al.*, 2004). Similarly, other terpenoids such as the monoterpenes may also function as antioxidants (Loreto *et al.*, 2004).

In another study the protective role of isoprene was linked to mediating thermotolerance of photosynthesis (Sharkey *et al.*, 2001). Fosmidomycin inhibition had no initial effects on photosynthesis. A quick high temperature treatment, however, showed an impaired recovery in fosmidomycin treated leaves in relation to normal leaves. The same study illustrated how isoprene and butadiene could increase thermotolerance in *Phaseolus vulgaris* L., a plant which does not produce isoprene itself. Molecular simulations have shown that isoprene may indeed stabilize membranes and aid in resisting a phase transition of membranes with an increase in temperature (Siwko *et al.*, 2007).

2.3.4 Volatile defence against biotic stress

Upon infection of oak trees (*Quercus fusiformis* Small.) with wilt (*Ceratocystis fagacearum* (T. W. Bretz) J. Hunt) the emission of isoprene is greatly reduced (Anderson *et al.*, 2000). The reduction was attributed to a lower rate of photosynthesis and by extension decreased

availability of carbon for the synthesis of isoprene. A similar reduction of isoprene was observed in *Melampsora epitea* Thümen. infected willow saplings (*Salix* sp. hybrid) but in turn an increased emission of monoterpenes (particularly β -ocimene), sesquiterpenes and LOX pathway products was observed (Toome *et al.*, 2010). The release of these volatiles at 6 and 12 days post infection (dpi) could be correlated to disease progression of *M. epitea*, as pustules become visible at 5 dpi and necrotic lesions at 11 dpi. The argument was made that the decrease of isoprene emission may be partially attributed to isoprene being channelled to the synthesis of higher classes of terpenoids such as sesquiterpenes. An induction of a terpene synthase gene was indeed observed in poplar plants infected with another *Melampsora* sp. at 6 dpi (Azaiez *et al.*, 2009), supporting the induced synthesis and emission of higher class terpenoids upon infection.

A number of PAMP's have been shown to induce volatiles in *Medicago truncatula* Gaertn. with β -glucan inducing the greatest variety of unique volatiles (Leitner *et al.*, 2008). The PAMP's perceived by plants may therefore not only induce innate defence but also lead to the emission of volatiles. The role of induced volatile emissions upon infection alludes to a function in defence that was and still is being intensely researched.

2.3.4.1 Direct defences

Direct defence by volatiles is where the compound itself is detrimental to the growth of an organism (microbes particularly). Volatiles released upon infection of peanut plants with white mould (*Sclerotium rolfsii* Curzi.) have been proven to inhibit the growth of this pathogen (Cardoza *et al.*, 2002). Treating the fungus in a growth medium with synthetic equivalents of 3-octanone, (*Z*)-3-hexenyl acetate, linalool or methyl salicylate showed a general pattern of reduced growth. It was particularly linalool and methyl salicylate at higher concentrations that were effective against the white mould cultures. Similarly the rate of germination and hyphal length of *Botrytis cinerea* (De Bary.) Whetzel. was greatly reduced when exposed to high levels of GLV's (Kishimoto *et al.*, 2008). Plant volatiles from various classes (including hexanal, eugenol and carvone) also inhibited the mycelial growth and conidial germination of *Neofabraea alba* (E.J. Guthrie) Verkley (lenticel rot) on apple fruit (Neri *et al.*, 2009).

Shiojiri *et al.* (2006) used transgenic *Arabidopsis* to investigate the effect of altered levels of GLV's in the defence response. High levels of GLV's decreased the necrotic lesion size of *B. cinerea* while lower levels increased the lesion size as compared to wild-type *Arabidopsis*. Interestingly, a (Z)-3-hexenal treatment decreased lesion size in the wild-type plants to sizes comparable to that on the transgenic plants. It should be noted that this effect may be attributed to either the direct antifungal properties of GLV's or the possible induction of other defences by these compounds.

2.3.4.2 Indirect defences

Volatiles mediating indirect defence does not show antimicrobial activity, but play a role in attracting the natural enemy of the organism causing infestation. This type of interaction in an ecosystem is called a tritropic interaction and has received much attention in recent times.

Brouat *et al.* (2000) described how symbiotic ants (*Petalomyrmex phylax* Snellig.) constantly patrol the young developing leaves of *Leonardoxa africana* (Baill.) Aubrév., and how this may be correlated to GLV's. Measured GLV concentrations were much higher in young leaves relative to mature leaves, thus acting as possible signals. The ants, which act as a defensive mechanism against pathogenic insects, are seemingly being assigned to protect young leaves. Infestation by *Pieris rapae* L. larvae also typically induced the release of GLV's in *Arabidopsis* (Shiojiri *et al.*, 2006). Transformed plants which emitted high levels of GLV's (as described in section 2.2.4.1) were more attractive to the parasitic wasps that use the larvae as host.

Tritropic interactions have now been noted for numerous plants that include the attraction of parasitic wasps upon infestation of maize by *Spodoptera littoralis* Boisduval. caterpillars (Ton *et al.*, 2006), the attraction of aphid parasitoids upon infestation of broad beans by *Acyrtosiphon pisum* Harris. aphids (Guerrieri *et al.*, 2002) and the attraction of predatory mites upon infestation of tomato by *Tetranychus urticae* C. L. Koch. mites (Kant *et al.*, 2004). Interestingly the volatile mixtures that are emitted upon *T. urticae* infestation differ between plant species (Van den Boom *et al.*, 2004). In nine out of eleven infested plants studied, volatiles emitted differed from those of uninfected or mechanically damaged plants of the same species. This indicated that unique volatiles could lead to a highly specific tritropic interaction in species.

Van Wijk *et al.* (2008) tested the attraction of predatory mites to 30 different volatiles of which only some have been implicated in a tritropic interaction upon *T. urticae* infestation. Only octanol, *cis*-3-hexenol and methyl salicylate were significantly more attractive to spider mites, but these were still less attractive than the mixture of volatiles that were emitted by *T. urticae* infested plants. Furthermore predatory mites must associate a volatile mixture with prey otherwise uninfested plants are not considerably more attractive than infested plants. Predatory mites seemingly cannot distinguish between single unique volatiles but must associate volatile mixtures with *T. urticae* infested plants (Van Wijk *et al.*, 2008)

Schnee *et al.* (2006) illustrated that the *tps10* gene is induced in wheat upon *S. littoralis* infestation. The gene codes for terpene synthase, an enzyme that produces sesquiterpenes (a sub-class of terpenoids). *Arabidopsis* transformed with the *tps10* gene not only emits more terpenoid volatiles but also attracts more parasitic wasps as compared to wild-type *Arabidopsis*, thereby proving an indirect defence response. Such research illustrates the usefulness of modifying volatile emissions by genetic manipulation with the goal of improving plant defence.

2.3.4.3 Interplant signalling

For some time volatiles have been described as important in mediating so called plant-to-plant communication (Baldwin and Schultz, 1983). The theory maintains that volatiles induced by biotic factors may diffuse via the atmosphere to neighbouring plants to induce a defence response in these plants. The phrase “plant communication” however is deceptive and henceforth the interaction will be referred to as interplant signalling. Research on interplant signalling following herbivore damage predominates, but interplant signalling may also occur upon pathogenic infection.

The defence response against either infestation or pathogenic infection is obviously quite dissimilar, and so too the volatiles released upon the respective stress conditions. Peanut plants emitted different volatiles when infected with *S. rolfsii* compared to emissions upon beet armyworm (*Spodoptera exigua* Hübner.) infestation (Cardoza *et al.*, 2002). Simultaneous infection and infestation emitted a mixture of volatiles with unique components from both instances. In silver birch the emission of certain volatiles also differs, with methyl salicylate for example being released upon infestation but not upon infection (Vuorinen *et al.*, 2007). Therefore volatile signals may differ greatly between different stress conditions.

Heil and Ton (2008) argued that interplant signalling upon biotic stress is simply a side-effect of signalling between distal parts of plants and that volatiles may be true signals for systemic resistance. Park *et al.* (2007) proved that methyl salicylate is the signal for SAR by using combinations of wild-type, mutant and transformed tobacco in grafting experiments. Primary tissue infected with tobacco mosaic virus must have salicylic acid methyl transferase activity, and therefore the ability to produce methyl salicylate. If the activity was absent SAR was not induced in systemic uninfected tissue. Systemic tissue in turn must have intact methyl salicylate esterase activity to convert the volatile back to salicylate, otherwise SAR was also not induced. Methyl salicylate was also proven to be transported via the phloem to systemic tissue. The possibility of diffusion via air to systemic tissue could not be excluded due to the experimental setup.

In contrast the production of methyl salicylate is not required for the induction of SAR in *Arabidopsis*, thereby excluding both transportation via air or phloem (Attaran *et al.*, 2009). The argument is that salicylic acid is a downstream defence signal, and that methyl salicylate is predominately emitted into the atmosphere as an overflow to regulate the effects of salicylic acid. Therefore methyl salicylate may not be a systemic signal in all plant species, but rather methyl jasmonate or other lipid derived molecules being more likely candidates.

Transgenic *Arabidopsis* with increased jasmonic acid carboxyl methyl transferase activity constitutively induced 80 genes and repressed 83 genes (Jung *et al.*, 2007). The induced genes resulting from the elevated methyl jasmonate included the *PR* genes and other defence associated genes. Prior research has also shown that transgenic *Arabidopsis* with high methyl jasmonate emissions are more resistant to *B. cinerea* infection (Seo *et al.*, 2001). Conventional models however describe jasmonate (and derivatives like methyl jasmonate) as being associated with infestation, while salicylate (and methyl salicylate) is associated with infection (Heil and Ton, 2008).

Considering larger plants such as trees, HIPV's may be likely candidates for systemic signals seeing that adjacent leaves may not have a direct vascular connection. Frost *et al.* (2007) isolated and connected distal leaves with an air tube. Some leaves therefore had contact to volatiles of damaged leaves and others not. Leaf consumption by *Lymantria dispar* L. larvae was not significantly different between treatments. Leaves which had a tube connection did however have a primed release of terpenoid volatiles. Blueberry shrubs also induced HIPV's upon infestation with *L. dispar* larvae (Rodriguez-Saona *et al.*, 2009). Branches that were not

exposed to HIPV's of infested branches were consumed in greater quantities relatively to exposed branches. The study therefore alludes to priming of defences.

Whether or not volatiles may be systemic signals remain to be seen, but the ability of volatile treatments to induce a defence response prior to infestation/infection is well established. Tschardtke *et al.* (2001) observed volatile signalling between black alder trees upon infestation with leaf beetle and investigated possible signals. Methyl jasmonate, methyl salicylate and ethylene all induced proteinase inhibitor activity and increased phenolic content, with ethylene having the most pronounced effect. Methyl jasmonate in turn significantly induced catalase activity. Ethylene have also been implicated to act synergistically with GLV's such as (Z)-3-hexanol (Ruther and Kleier, 2005).

A methyl jasmonate treatment was shown to induce the transcription of numerous *PR* genes and by extension delayed the subsequent growth of crown rot (*Fusarium pseudograminearum* O'Donnell and T. Aoki) on wheat (Desmond *et al.*, 2006). Treating wheat with methyl jasmonate also visibly reduced the damage caused by leaf rust (*P. triticina*) with a number of defence mechanisms (including chitinase activity) being induced (Haggag and Abd-El-Kareem, 2009). Such a volatile treatment may support interplant signalling and identify possible signals but does not in itself prove the existence of such an event.

Infection of maize with various *Fusarium* spp. resulted in the increased emission of GLV's, terpenoids, methyl salicylate and other benzenoid volatiles (Piesik *et al.*, 2011b). Uninfected maize plants growing in close proximity also induced the release of volatiles, and more so plants growing one meter away in comparison to those three meters away. Similar results were observed by Wenda-Piesik *et al.* (2010) in wheat infected with various *Fusarium* spp. which could induce a volatile release in uninfected wheat, barley or oats. Again the effects decreased if uninfected plants were placed at a greater distance away from infected plants. The role of emitted volatiles in uninfected plants was not described and could only be speculated upon.

An excellent case for interplant signalling has been made by Karban *et al.* (2000) which also illustrated that the phenomenon may occur between species. In the study tobacco plants obtained greater resistance against insect herbivory when grown in close proximity to clipped sagebrush relative to plants grown close to unclipped sagebrush. The study was done in an open field and the possibility that the signals travelled through the atmosphere is most

probable. Further investigation showed that tobacco must at most be 10 cm away from clipped sagebrush for the signalling to occur (Karban *et al.*, 2003).

In laboratory experiments simulating natural conditions, acceptance of aphids (*Rhopalosiphum padi* L.) by barley decreased when plants were exposed to air of infested barley (Pettersen *et al.*, 1999). The interaction has also been attributed to volatiles, but interestingly only for certain combinations of infected and uninfected (exposed) barley cultivars. Initial results from Karban and Shiojiri (2009) have also shown that herbivore damage was greater in sagebrush individuals exposed to volatiles from genetically dissimilar sagebrush. Interplant signalling was more pronounced between genetically identical sagebrush individuals (made from cuttings), thereby implying some manner of recognition of self and non-self via volatiles.

Gouinguéné *et al.* (2001) used gas chromatography/mass spectrometry to investigate variations in maize HIPV's released upon treatment with *S. littoralis* regurgitant. Eleven maize (*Zea mays* L.) cultivars and five related *Zea* spp. were compared, with variations being observed in both volatile amount and composition. The variation was especially large for certain terpenoid volatiles. Within a given *Z. mays* population there was some variation in the quantity of volatiles but no significant variation in composition. Variation may therefore also be present when comparing volatile signals and the ability of plants to perceive such a signal, as mentioned before (Pettersen *et al.*, 1999; Karban and Shiojiri, 2009). Variation may exist between different plant species and even different cultivars.

A high volatile concentration either naturally produced or as pure synthetic treatment induces a complete defence response. A lower concentration in turn simply primes the defence mechanisms (Conrath *et al.*, 2006; Heil and Ton, 2008). In such a primed state the induced defence mechanisms arise more rapidly and/or intensely in response to infection or infestation (Conrath *et al.*, 2006). Therefore a preceding induction of defence by volatiles in the absence of any other elicitation may not be observable at concentrations naturally present.

Ton *et al.* (2006) illustrated priming by volatiles in both direct and indirect defence. The study used maize plants infested with *S. littoralis* to expose uninfested plants to its emitted volatiles in a continuous flow system. The induced expression of six defence genes was primed in the exposed plants. Maize plants exposed to volatiles and treated with an elicitor also produced greater levels of GLV's and terpenoids. These plants were also more attractive

to parasitic wasps in a tritropic interaction. The growth of *S. littoralis* larvae was also retarded when feeding on the exposed plants. The weight comparison of the larvae was done at 11 hours post exposure (hpe), but longer periods of infestation had less significant differences.

Frost *et al.* (2008) showed that the GLV *cis*-3-hexenyl acetate also primed the defence response in hybrid poplar trees against *L. dispar* larvae. A treatment of plants with this volatile primed the release of jasmonate, linolenic acid and certain terpenoids. Furthermore, genes that were primed with the treatment included those coding for a lipoxygenase, a proteinase inhibitor and an enzyme involved in the synthesis of antifungal phytoalexins. All of the above mentioned variables were significantly different at 24 hours after infestation, but similar at 48 hours. The effects of priming by volatiles may therefore diminish with time but still gives a competitive advantage during early stages of infestation/infection.

Other GLV's have also been implicated as priming agents. Maize seedlings were primed by treatment with either (*Z*)-3-hexenal, (*Z*)-3-hexenol or (*Z*)-3-hexenyl acetate (pure synthetic GLV's) (Engelberth *et al.*, 2004). Similar to either a mixed GLV treatment or natural volatiles released from infested plants, these volatiles were shown to induce jasmonic acid synthesis and volatile emissions in undamaged plants. However, once *S. exigua* regurgitant was applied (simulating infestation), both of the above mentioned variables were induced to even greater levels.

Interplant signalling was also proven in lima beans where untreated plants were exposed to volatiles from plants with artificially induced SAR (using a benzothiadiazole treatment) (Yi *et al.*, 2009). After exposure the untreated plants were infected with *P. syringae* and a primed induction of *PR2* and decreased lesion number relative to unexposed plants were observed. Methyl salicylate and nonanal were identified as candidates for volatile signals from SAR-induced plants. A nonanal treatment induced *PR2* and *LOX* expression and primed the expression of both. Methyl salicylate however induced only *PR2* and primed only *LOX*. A nonanal treatment also decreased the pathogen population over a three day period.

One might speculate that volatile emissions upon infestation/infection are predominately a side-effect of the stress condition. No competitive advantage may be envisioned for volatiles released into the atmosphere that are not involved with defence (direct or indirect), and therefore are essentially a net loss of assimilated carbon. The true competitive advantage may

be the perception of volatiles and priming of defences by a neighbouring plant. Further research must be done to prove interplant signalling and the exact conditions whereby the phenomenon occurs. The ultimate goal of research is the manipulation of interplant signalling for obvious commercial value.

The ideal setup for experimentation in interplant signalling must expose uninfested/uninfected plants to a mixture of volatiles from a natural source. Accomplishing this in a laboratory or glasshouse may be difficult and leads to relevant criticism. Investigation of volatile effects using an enclosed experimental system is ultimately flawed as such a system may allow volatile compounds to increase to levels not present in the natural environment (Dicke *et al.*, 2003; Paschold *et al.*, 2006). Experimental conditions must therefore emulate natural conditions and signalling via the atmosphere must be exclusively confirmed. Systems have been designed to allow for continuous air-flow between plants and have been used to investigate various aspects of plant volatile signalling (Pettersen *et al.*, 1999; Ninkovic *et al.*, 2002; Ninkovic, 2003; Paschold *et al.*, 2006; Ton *et al.*, 2006). Figure 2.1 is an example of such a system which allows air-flow from the affected plant towards unaffected plants that does not cause the accumulation of volatiles. The setup is similar to natural conditions where volatiles are released into the atmosphere and the dispersal to neighbouring plants are dependent on external factors such as wind.



Figure 2.1: Continuous air-flow chamber used for volatile exposure experiments based on the original design of Petterson *et al.* (1999). The photo was received by personal communication with V. Ninkovic (Swedish University of Agricultural Sciences).

Chapter 3: Materials and methods

3.1 Materials

3.1.1 Biological material

Different wheat (*T. aestivum*) lines resistant (Thatcher+*Lr9*, RL 6010) and susceptible (Thatcher) to leaf rust infection were used in this study. Leaf rust (*P. triticina*) pathotype UVPt9 was used for all infection studies. UVPt9 has an incompatible interaction with Thatcher+*Lr9* and a compatible interaction with Thatcher. Thereby allowing the comparison of interplant signalling in resistant and susceptible near-isogenic lines. The pathogen was multiplied on the susceptible Karee cultivar.

3.1.2 Continuous air-flow chamber design for volatile exposure of wheat

The design of the continuous air-flow chamber (figure 3.1) was based on that of Petterson *et al.* (1999) with minor modifications. The chamber was made of Perspex and designed to allow placement of seedlings planted in cones, from the top. Air was drawn through the system using an extractor fan to allow movement of air from the infected and mock infected resistant and susceptible plants towards the experimental plants (figure 3.2). No contact was possible between the two large compartments. Furthermore, individual cones had no contact via either the soil or water (figure 3.3). Before placing the cones in the system, an equal volume of water was added to each cone holder.

3.2 Methods

3.2.1 Cultivation and infection of wheat

Wheat seedlings were germinated and grown under rust free conditions in a glasshouse at 18-25°C where a 14 h light and 10 h dark cycle was maintained with additional light supplied by cool fluorescent lights with photosynthetically active radiation of 120 $\mu\text{mol}/\text{m}^2/\text{s}$. Six seeds were planted in each plastic cone containing a sterilized 1:1 peat/soil mixture. Multifeed P fertilizer (Plaaskem) (N:P:K ratio 5:2:4) at a concentration of 8 g/l was given two days prior and five days post infection.

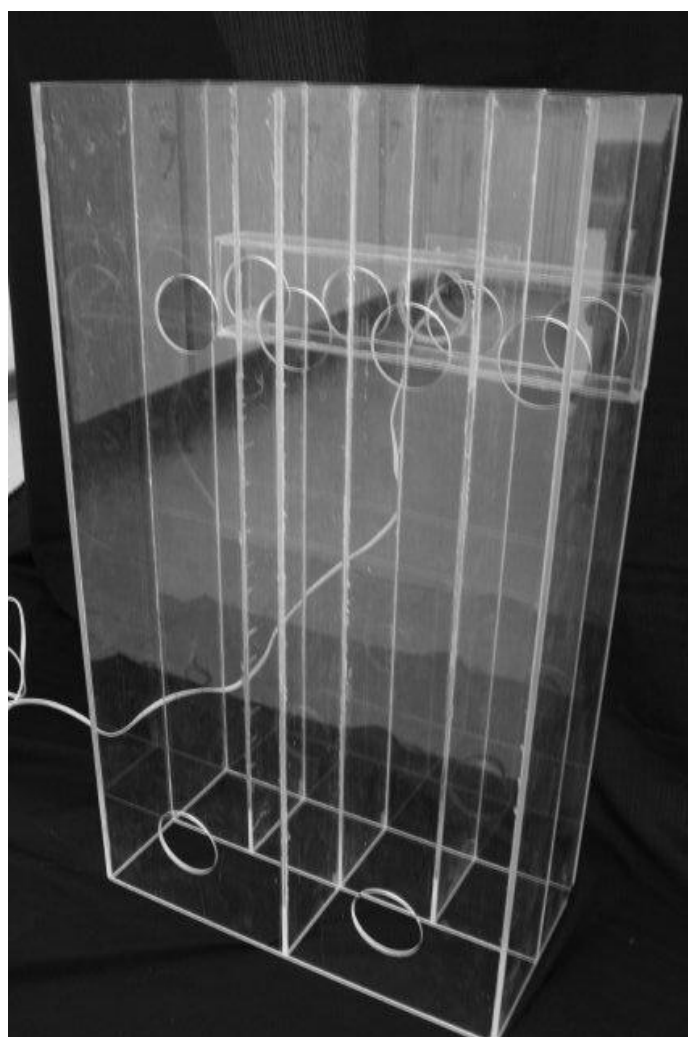


Figure 3.1: The constructed continuous air-flow chamber designed according to Petterson *et al.* (1999). The set-up was used for volatile experiments with constant air-flow generated by an extractor fan at the far rear.

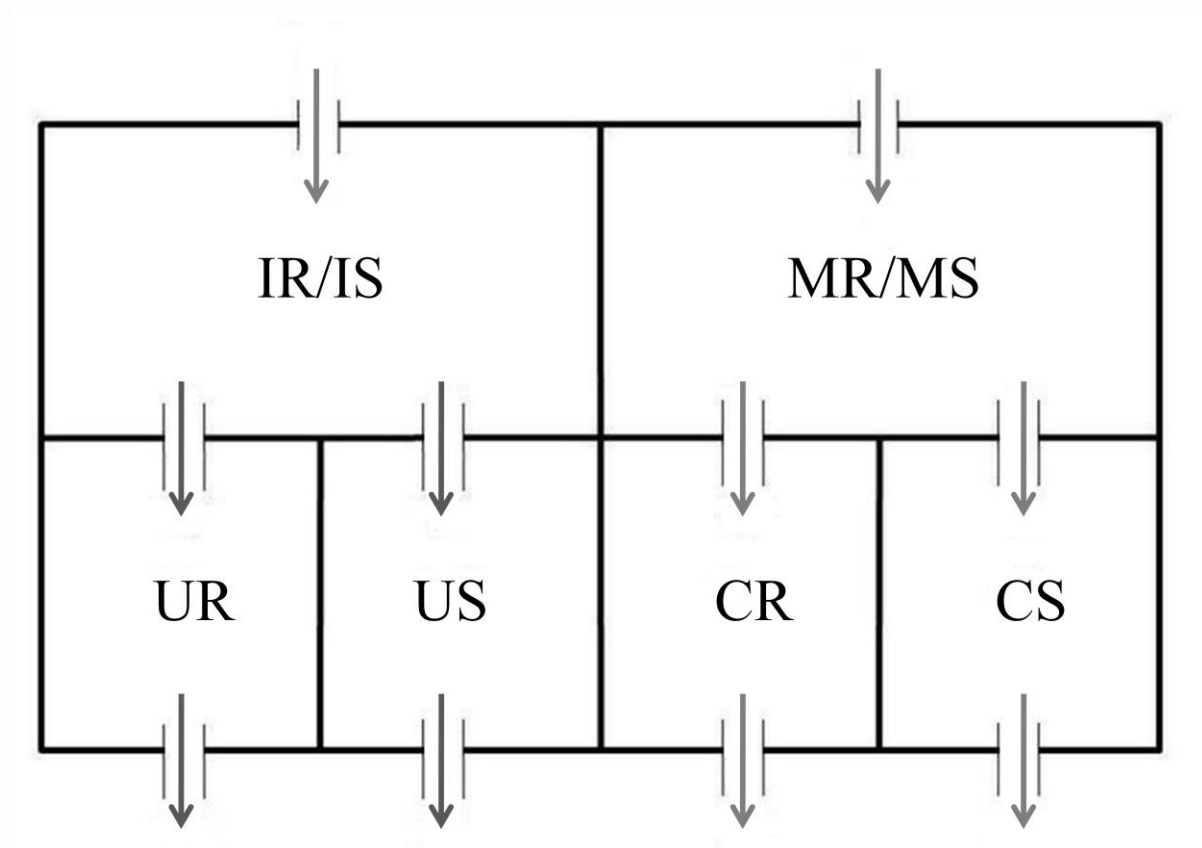


Figure 3.2: Schematic representation of the experimental setup for volatile exposure of seedlings. Arrows indicate the direction of air-flow. Seedlings in the chamber divisions correspond to infected resistant (IR), infected susceptible (IS), mock infected resistant (MR), mock infected susceptible (MS), uninfected resistant (UR), uninfected susceptible (US), control resistant (CR) and control susceptible (CS).

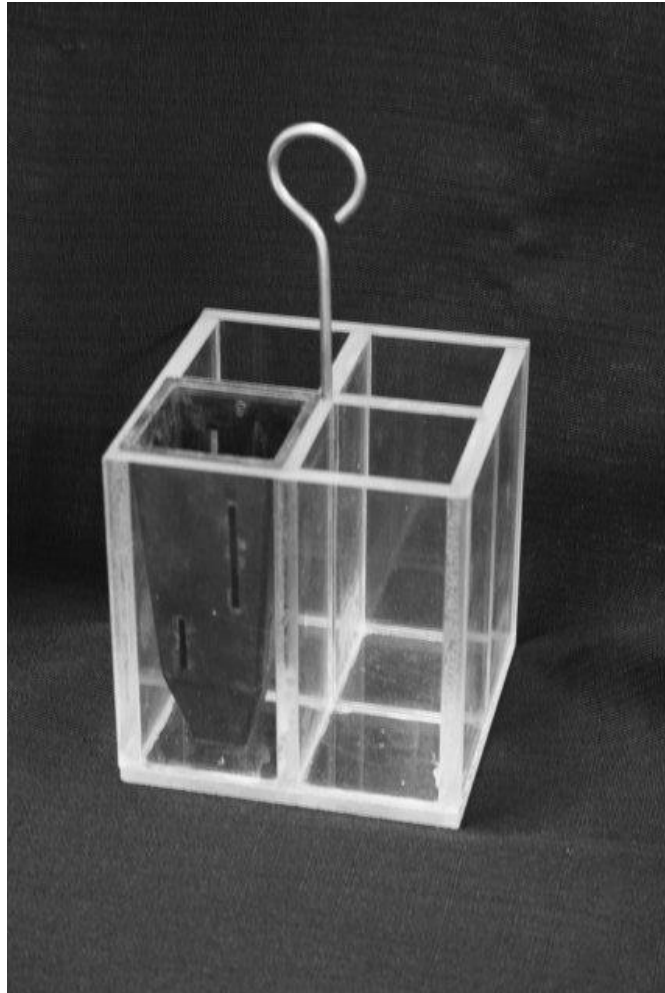


Figure 3.3: Perspex holder for four cones containing wheat seedlings.

The susceptible cultivar Karee was used for the multiplication of leaf rust urediospores. Five day old Karee seedlings were treated with 0.3 g/l maleic hydrazide to slow down their growth. After ten days, seedlings were infected with a concentrated *P. triticina* urediospore solution. Urediospores harvested and stored at -80°C were heat shocked at 48°C for 6 min, resuspended in kerosene oil and sprayed under pressure onto seedlings. After drying off the plants for 2 h at 20°C, seedlings were incubated for 16 h in the dark at 20-23°C under high humidity to allow infection to occur. Before transferring the seedlings back to the glasshouse, they were again dried for 2 h at 20°C.

For experimental infection, freshly harvested *P. triticina* urediospores were suspended in kerosene oil at a concentration of 1 mg/ml and 800 µl of the spore suspension was sprayed onto eight seedling cones. The mock infected plants were sprayed with kerosene oil containing no spores. Using kerosene oil as inoculum medium and subsequent humid incubation may well cause a response in seedlings; the treatment however was present in both the experimental and control seedlings.

3.2.2 Volatile exposure experiments

A diagrammatical time-line of the volatile experiments is presented in figure 3.4. Four cones containing ten day old resistant or susceptible seedlings were either mock infected or infected with leaf rust. These plants were used as volatile source at 5 dpi when four cones containing six 10 day old uninfected resistant or susceptible seedlings each were exposed to the released volatiles. Prior to exposure, six seedlings representing time zero was harvested.

During the first experiment uninfected resistant (UR) and susceptible (US) seedlings were exposed to infected resistant (IR) seedlings. Control resistant (CR) and susceptible (CS) seedlings were exposed to mock infected resistant (MR) plant volatiles. UR, US, CR and CS were exposed to the respective volatile source plants simultaneously, but the experimental and control seedlings had no contact via air currents.

The second experiment had a fundamentally similar setup to the first but the source of volatiles differed. Here the experiment consisted of UR and susceptible US plants that were exposed to infected susceptible (IS) seedlings. In parallel control seedlings were exposed to volatiles released by mock infected susceptible (MS) plants.

Both the above mentioned experiments were done independently in triplicate. During the 24 h exposure, a single cone was harvested at 8 and 24 hpe respectively and the tissue immediately frozen in liquid nitrogen. Tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle and stored at -80°C. The tissue was used for enzyme activity and gene expression analysis.

The last remaining two cones that were not harvested after volatile exposure were subsequently infected with leaf rust urediospores (UVPt9).The infected seedlings were incubated in the glasshouse for 10 days where after it was photographed. All UR and US treatments are subsequently referred to as later infected resistant (LIR) and susceptible (LIS). Likewise CR and CS plants were also infected. As a further control, 10 day old resistant and susceptible seedlings not exposed to any volatiles were similarly infected and photographed (referred to as IR and IS).

3.2.3 Phenotypical analysis of volatile exposed and control plants

Photographs of infected volatile exposed and control plants were analyzed using Assess Image Analysis Software for Plant Disease Quantification (supplied by the American Phytopathological Society, Saint Paul, Minnesota, USA). Phenotypical differences of the resistant seedling treatments could not be quantified using the software but were still compared on a visual basis. The differences between the various treatments of the susceptible plants (LIS, CS and IS) were determined as percentage area affected by leaf rust and average size of rust pustules. A minimum of 10 individual leaves per treatment were used. An analysis of variance and a Tukey's multiple comparison test was used to distinguish between means. Statistical analysis was done with GraphPad Prism 5.02 (La Jolla, California, USA).

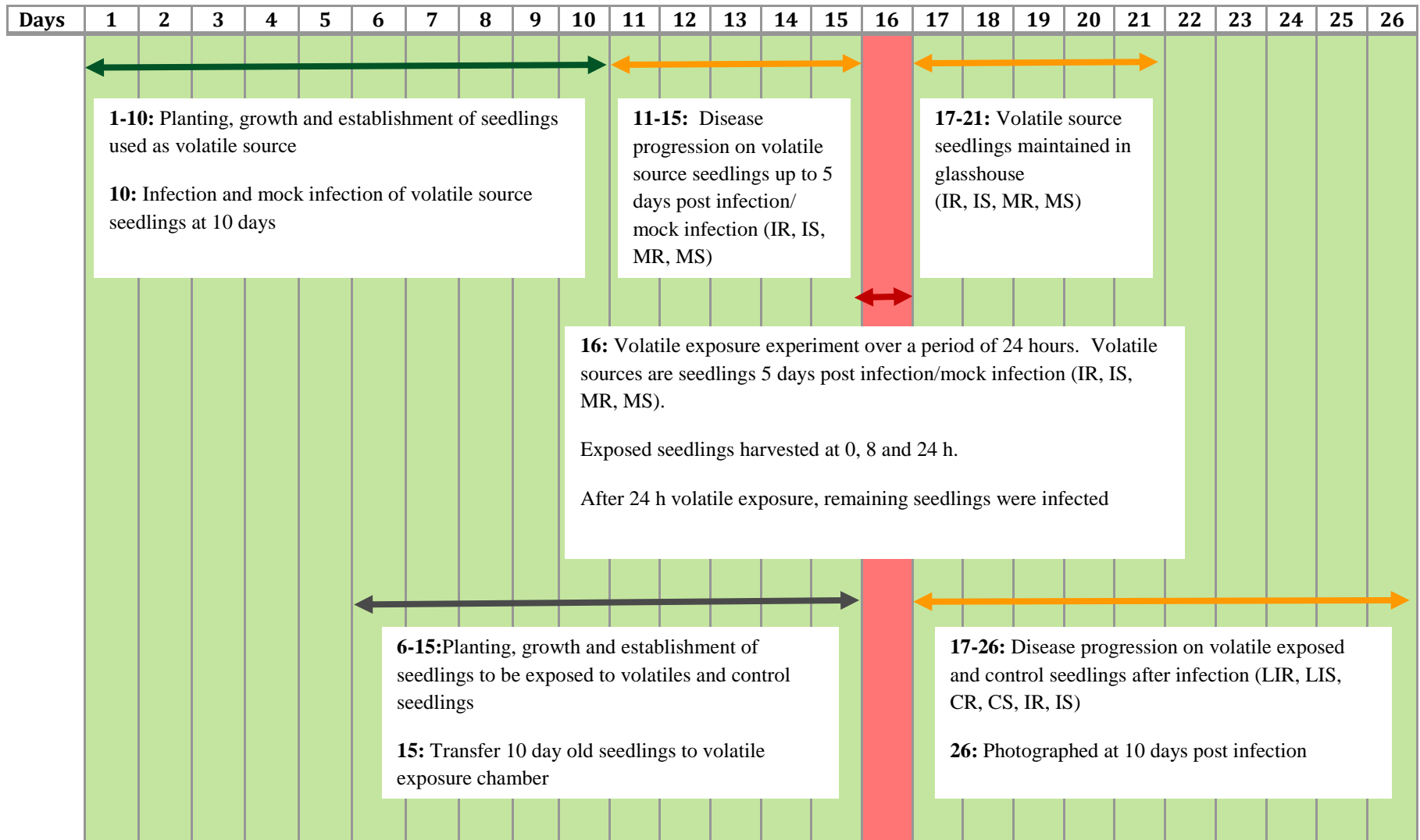


Figure 3.4: Schematic time-line representation of volatile exposure experiments. Days are indicated as relative to the first day of planting wheat seedlings. Green arrows represent uninfected plants and orange arrows infected plants. Volatile exposure using the designed chamber occurred on the day indicated with the red arrow.

3.2.4 Biochemical analysis of volatile exposed and control plants

3.2.4.1 Protein extraction

Total protein was extracted from approximately 100 mg frozen tissue by adding an extraction buffer in a ratio of 1:4. The extraction buffer contained 10 mM β -mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM phenylmethanesulfonyl fluoride (PMSF) in a 50 mM tris(hydroxymethyl)aminomethanehydrochloric acid (Tris-HCl) pH 7.5 buffer. After the suspension was vortexed, it was centrifuged at 10 000 *g* for 10 min at 4°C. The clear supernatant was frozen in aliquots at -20°C.

Protein concentration was determined using the BioRad (Hercules, California, USA) Protein Assay described by Bradford (1976). The absorbance was read at 595 nm using the Zenyth 3100 Multimode detector (Anthos Labtec, Salzburg, Austria). All measurements were performed in quadruplicate. For the standard curve, γ -globulin (Sigma Aldrich, Saint Louis, Missouri) was serially diluted and the absorbance determined. Protein concentration of the samples was determined using the γ -globulin protein concentration versus absorbance standard curve (figure 3.5).

3.2.4.2 Determination of β -1,3-glucanase activity

β -1,3-glucanase activity was selected as a marker of induced defence and determined using a modified method of Fink *et al.* (1988). Each reaction contained 20 μ l total protein extract, 500 μ l laminarin (2 mg/ml; Sigma, Saint Louis, Missouri, USA) and 480 μ l 50 mM sodium acetate buffer pH 4.5. After incubation for 10 min at 37°C, 1 ml Somogyi's reagent (Somogyi, 1952) was added. The mixture was incubated for another 10 min at 100°C where after 1 ml Nelson's reagent (Nelson, 1944) was added. The absorbance was then measured at 540 nm using a Varian (Palo Alto, California, USA) Cary 100 Spectrophotometer. For the standard curve, known concentrations of glucose were treated accordingly and the glucose concentration plotted against absorbance on a graph to determine the linear relationship (figure 3.6). All measurements were performed in quadruplicate. The amount of glucose in each experimental sample was determined using the prepared standard curve and activity expressed as μ g glucose/ μ g protein. The blank reaction contained no total protein extract.

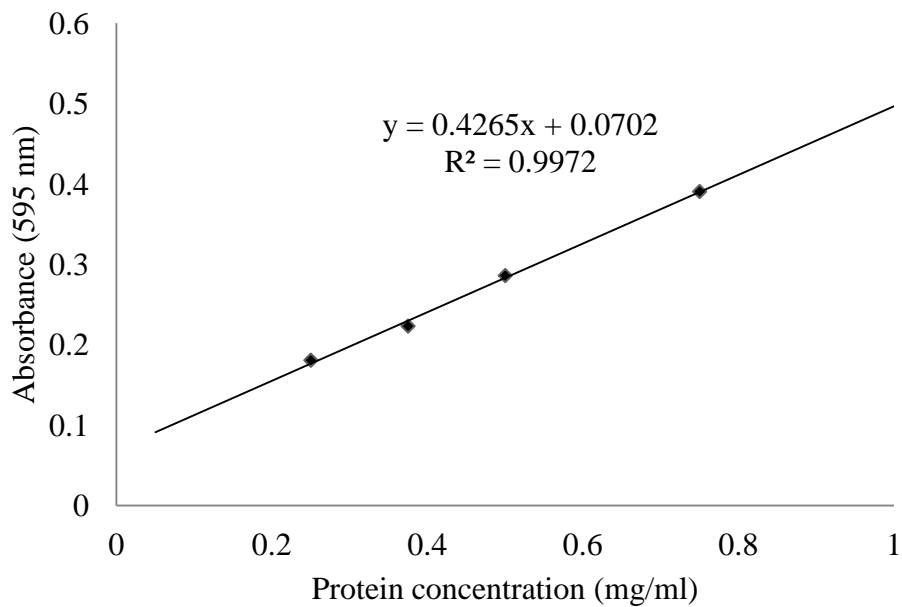


Figure 3.5: Standard curve for protein concentration determination. Curve shows absorbance (at 595 nm) versus protein concentration (mg/ml) of a γ -globulin standard as determined by the method of Bradford (1976). Protein concentrations of unknown samples were extrapolated from the standard curve using measured absorbance values.

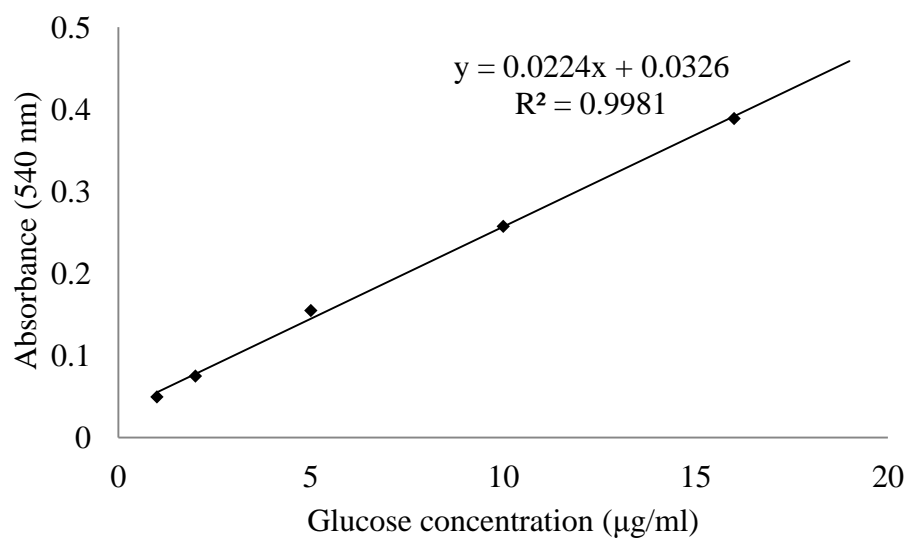


Figure 3.6: Standard curve for glucose concentration determination. Curve shows absorbance (at 540 nm) versus glucose concentration ($\mu\text{g/ml}$) as determined by the method of Fink *et al.* (1988). Glucose concentrations of unknown samples were extrapolated from the standard curve using measured absorbance values.

3.2.5 Expression analysis of volatile exposed and control plants

3.2.5.1 Total RNA extraction from wheat tissue

RNA extraction, dilution and experimentation were done using ribonuclease free water. The ribonuclease free water was prepared by adding 0.1% (v/v) dimethyldicarbonate (DMDC) to deionised water and leaving it overnight at room temperature. The water was finally autoclaved twice to destroy the DMDC.

Total RNA was extracted from 100 mg wheat leaf tissue using TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, California, USA) (Chomczynski and Sacchi, 1987). After finally dissolving the RNA in 200 µl DMDC water, the concentration was determined using the NanoDrop 200 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and the concentration expressed as ng/µl. RNA quality was assessed by separating 200 ng RNA on a 1% (w/v) denaturing agarose gel (Sambrook *et al.*, 1989) prepared in a 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA). The RNA was dissolved in 1x MOPS buffer, 50% (v/v) formamide, 6.5% (v/v) formaldehyde and 50 µg/ml ethidium bromide, denatured at 65°C for 15 min and separated using 1x MOPS running buffer. The gel was photographed under ultraviolet light illumination (302 nm) using the GelDoc XR+ System (BioRad, Hercules, California, USA).

3.2.5.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

The *PR2* gene was selected as a marker of induced defence and gene expression determined using the RobusT II RT-PCR kit (Finnzymes/Thermo Scientific, Woburn, Massachusetts, USA). The constitutively expressed *actin* reference gene was used as control. Each 10 µl RT-PCR reaction contained 10 ng total RNA, 10 pmol of each primer (Table 3.1), 1.5 mM MgCl₂, 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 1x RobusT reaction buffer and 0.4 µl M-MuLV reverse transcriptase and DyNAzyme DNA polymerase respectively. The amplification regime was 48°C for 30 min, 94°C for 1 min, 30 cycles of 94°C for 15 sec, specific annealing temperature (Table 3.1) for 15 sec and 72°C for 1 min. A final step of 7 min at 72°C was included. The RT-PCR reactions were separated on a 1% (w/v) agarose gel (Sambrook *et al.*, 1989) prepared in 0.5x TAE (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.28% (v/v) acetic acid) containing 0.5 µg/ml ethidium bromide and the gel photographed.

Table 3.1: Nucleotide sequence and annealing temperature for primer pairs used during the study.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')	T _a (°C)
<i>Actin</i>	AACAGAGAGAAGATGACCCAA	CGCACTTCATGATGGAGTTGT	61.2
<i>PR2</i>	CTCGACATCGGTAACGACCAG	GCGGCGATGTACTTGATGTTC	52.8

3.2.6 Capture and analysis of volatiles emitted by infected and mock infected seedlings

3.2.6.1 Solid phase micro-extraction (SPME) of volatiles

Approximately 30 seeds of both resistant and susceptible wheat lines were planted in separate pots. Ten day old seedlings were infected with *P. triticina* (UVPt9) urediospores at a concentration of 3 mg/ml in kerosene oil as described (3.2.1). Likewise, resistant and susceptible seedlings were mock infected with kerosene oil containing no spores. After 5 days, seedlings were enclosed in a 250x 300mm oven bag with a rubber band securing the outside of the bag to the pot. Before use, the oven bags were baked at 100°C for 2 h to remove possible contaminating volatile organic compounds. A septum was added to the top of the oven bag and the whole system sufficiently sealed. The needle of the SPME assembly was gently pushed through the septum and the 2cm x 30µm fibre (Divinylbenzene/Carboxen/Polydimethylsiloxane; Supelco, Saint Louis, Missouri, USA) was exposed to volatiles in the sealed system for 24 h. The SPME fibre was reconditioned at 250°C for 1 h before each use.

3.2.6.2 Gas chromatography mass spectrometry (GC/MS) analysis of volatiles

GC/MS analysis was carried out on a Finnigan Trace GC ultra/Finnigan Trace DSQ apparatus (West Chester, Ohio, USA). The instrument was equipped with a Varian (Palo Alto, California, USA) FactorFour 5MS capillary column (95% dimethylpolysiloxane, 5% phenyl; 30 m x 0.25 mm x 0.25 µm). Helium at a constant flow (1.0 ml/min) served as carrier gas. Desorption of samples occurred in the injection port at a temperature of 250°C for 2 min. Samples were injected with a splitless mode for 2 min, thereafter at a split ratio of 1:10. The injector temperature and transfer line were set at 250°C. Separation of volatile compounds was achieved under programmed conditions (40°C for 5 min, 4°C/min to 200°C, and 200°C for 2 min). The mass spectrum scan range was 40-500 au and the ion source temperature was set at 200°C. Initial data analysis was done on the Xcaliber 1.4 (Thermo Electron Corporation, Waltham, Massachusetts, USA) programme. Data was exported to the TurboMass 5.4.2 (PerkinElmer, Waltham, Massachusetts, USA) for final analysis. Compounds were identified by matching the spectra against reference spectra on the National Institute of Standards and Technology database.

Chapter 4: Results

4.1 Wheat infection

Resistant and susceptible wheat seedlings were infected/mock infected when 10 days old. At 5 dpi these plants were used as sources of volatile emissions for a 24 hour period in a continuous air-flow chamber. At this stage visible infection symptoms only started to appear. These seedlings were therefore kept within the glasshouse for a further 5 days to confirm successful infection (figure 4.1). Both mock infected seedlings showed no visible pustules forming on the surface of the leaves. The IR seedlings showed no visible pustules, but only some necrotic spots that were indicative of a typical HR response. The IS seedlings in turn however showed a prolific spread of rust pustules, confirming its susceptible phenotype.

4.2 Phenotypical analysis of volatile exposed and control wheat seedlings

To investigate the influence of emitted volatiles on leaf rust development in exposed seedlings, representative infected seedling leaves exposed to IR, MR, IS and MS seedlings were photographed (figure 4.2). When infected resistant and susceptible seedlings were respectively used as volatile sources, LIS seedlings showed a less severe response/spread of leaf rust pustule growth compared to the IS seedlings.

To accurately quantify these phenotypic differences, 10 to 12 infected leaves for all treatments were randomly selected and quantitatively analyzed (figures 4.3 and 4.4). The two parameters considered were percentage leaf area affected and relative pustule size. Analysis of variance indicated that treatment means differed significantly. The phenotypic analysis could only be done on susceptible seedlings since the HR response in resistance seedlings could not be accurately quantified by the software.

For susceptible seedlings exposed to volatiles emitted by resistant seedlings, there was a marked difference in percentage leaf area affected (figure 4.3a). LIS had the lowest value relative to IS seedlings, while CS seedlings were intermediate to both the LIS and IS leaf phenotypes. LIS, CS and IS all differed significantly from one another with regard to percentage area affected as determined by a Tukey's multiple comparison test. When the relative pustule size was considered, LIS and CS did not differ significantly from each other, but both were significantly lower than IS (figure 4.3b).

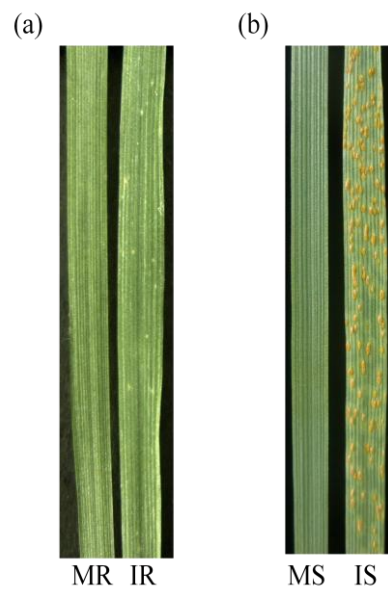


Figure 4.1: Infection of susceptible Thatcher and resistant Thatcher+*Lr9* wheat with leaf rust race UVPt9. Symptoms are visible on representative leaves of (a) mock infected resistant (MR) and infected resistant (IR) and (b) mock infected susceptible (MS) and infected susceptible (IS) wheat seedlings 10 dpi.

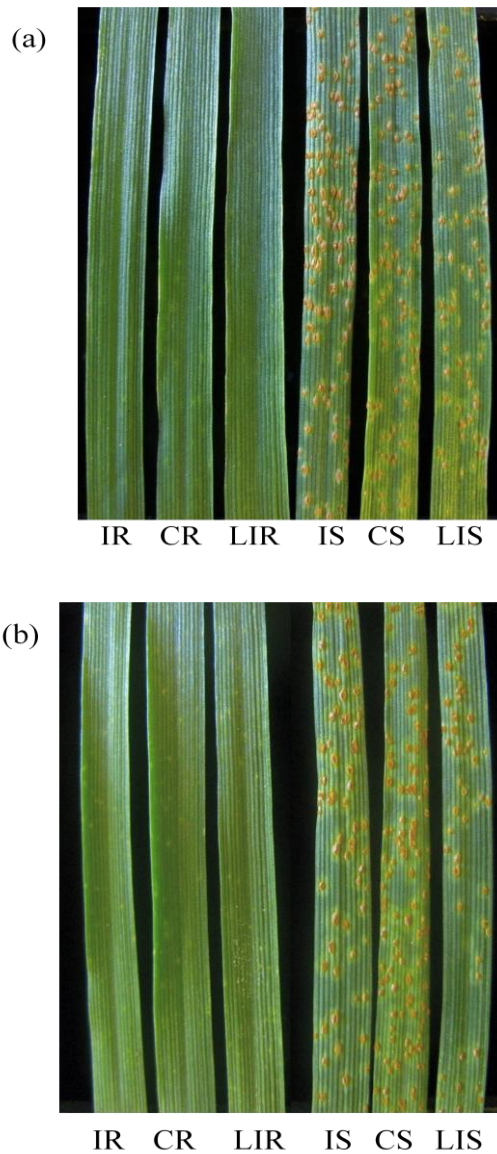


Figure 4.2: Influence of emitted volatiles on leaf rust development on exposed and infected resistant and susceptible wheat seedlings. Photographs were taken 10 dpi and indicate the representative phenotypic response from a number of seedlings.

(a) Infected resistant (IR) and infected susceptible (IS) seedlings were exposed to ambient glasshouse volatile emissions. Control resistant (CR) and control susceptible (CS) were exposed to mock infected resistant (MR) plant volatiles. Later infected resistant (LIR) and later infected susceptible (LIS) seedlings were exposed to infected resistant (IR) plant volatiles.

(b) Infected resistant (IR) and infected susceptible (IS) were exposed to ambient glasshouse volatile emissions. Control resistant (CR) and control susceptible (CS) were exposed to mock infected susceptible (MS) plant volatiles. Later infected resistant (LIR) and later infected susceptible (LIS) seedlings were exposed to infected susceptible (IS) plant volatiles.

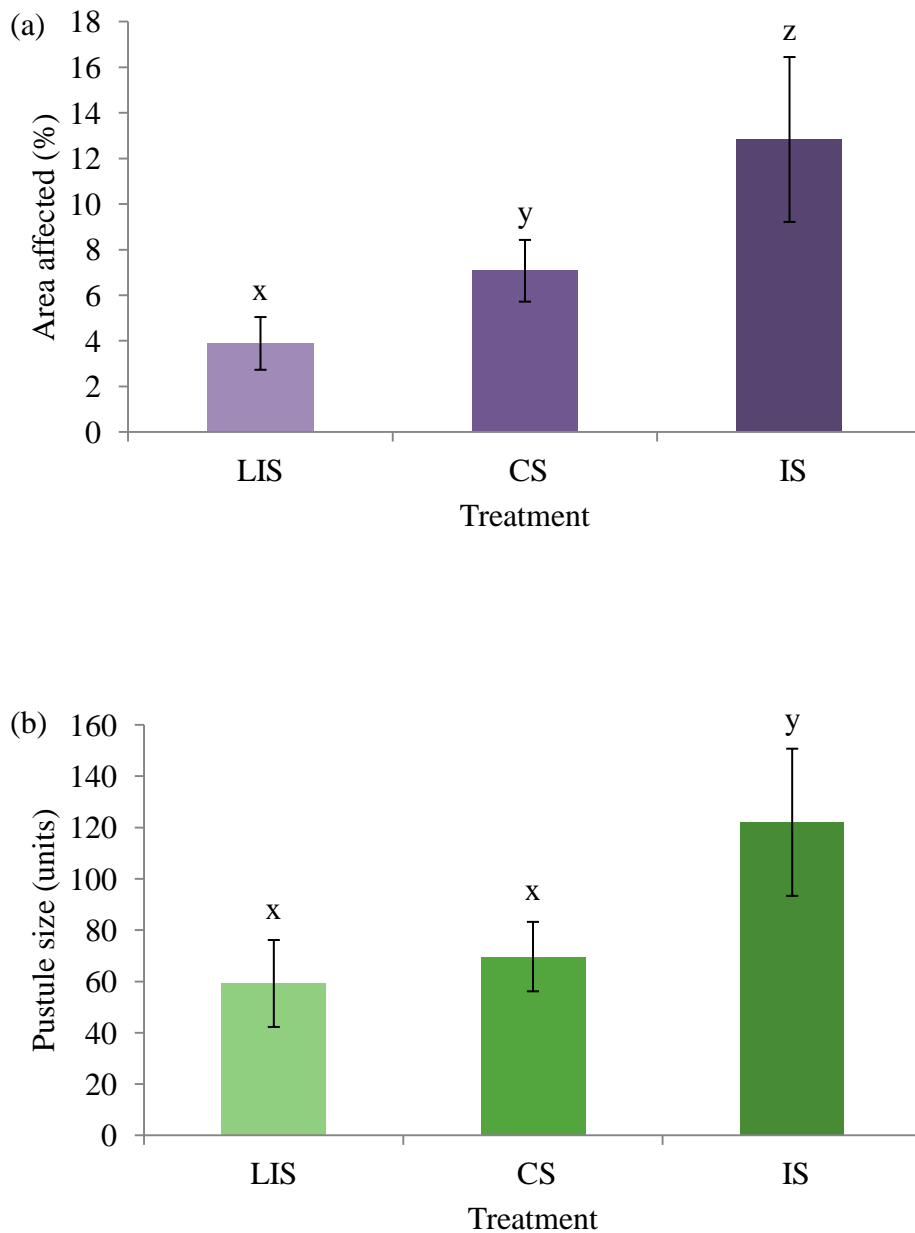


Figure 4.3: Leaf rust development in infected susceptible wheat seedlings after exposure to volatiles emitted by resistant seedlings. (a) Percentage area affected and (b) relative pustule size were calculated from photographs taken at 10 dpi of susceptible wheat seedling leaves. Measurements were determined for later infected susceptible (LIS), control susceptible (CS) and infected susceptible (IS) seedlings exposed to either IR, MR or typical glasshouse volatile emissions respectively. Treatment means with different letters differ significantly ($P < 0.05$) as determined by a Tukey's multiple comparison test. $n=12$ leaves.

When exposed to volatiles emitted by susceptible seedlings, the differences were less pronounced (figure 4.4). Significant difference in means was again determined by a Tukey's multiple comparison test. The percentage area affected in LIS differed significantly from IS, but not CS which in turn did not differ significantly from IS (figure 4.4a). Taking average relative pustule size into consideration, both LIS and CS were significantly different from IS, but could not be distinguished from each other (figure 4.4b).

4.3 β -1,3-Glucanase activity in volatile exposed and control wheat seedlings

To confirm the activation of a defence response in the volatile exposed plants, the enzyme activities of three different PR proteins were determined. Since this was done using total extracted protein instead of apoplastic protein, chitinase and peroxidase activity levels were too low to detect (results not shown). β -1,3-glucanase activity however was determined over a period of 24 h in exposed and control seedlings and expressed as $\mu\text{g glucose}/\mu\text{g protein}$. It should be noted that the activities were lower than expected.

Two of the triplicate β -1,3-glucanase activity analyses of seedlings exposed to resistant seedlings (figure 4.5a and b) showed a similar induction pattern over time. Enzyme activity increased from 0 hpe to a maximum at 8 hpe but decreased toward 24 hpe. The remaining replicate (figure 4.5c) also showed an induction of enzyme activity but peaking at 24 hpe. Both the experimental (UR and US) and control (CR and CS) samples showed the induction of β -1,3-glucanase activity in the first two replicates (figure 4.5a and b), whereas only the experimental samples showed induction in the third (figure 4.5c). However the induction in the experimental plants was always greater than that seen in the control plants.

In figure 4.5a, a 2.4 fold induction of activity was observed in UR plants at 8 hpe compared to a 1.6 fold induction in the CR plants. Correspondingly a 1.8 fold increase was observed in US relative to the 1.1 fold increase in CS for the same period of time. In figure 4.5b, a 4.1 fold induction of activity was noted in UR relative to a 1.8 fold induction in CR seedlings at 8 hpe. The US seedlings showed a 1.9 fold increase compared to a 2.1 fold increase for CS after 8 hpe. In figure 4.5c, a 3.4 fold induction of activity was observed in UR after 24 hpe with no increase in CR. Likewise US showed no induction after 24 hpe but CS was marked by a slight decrease in activity.

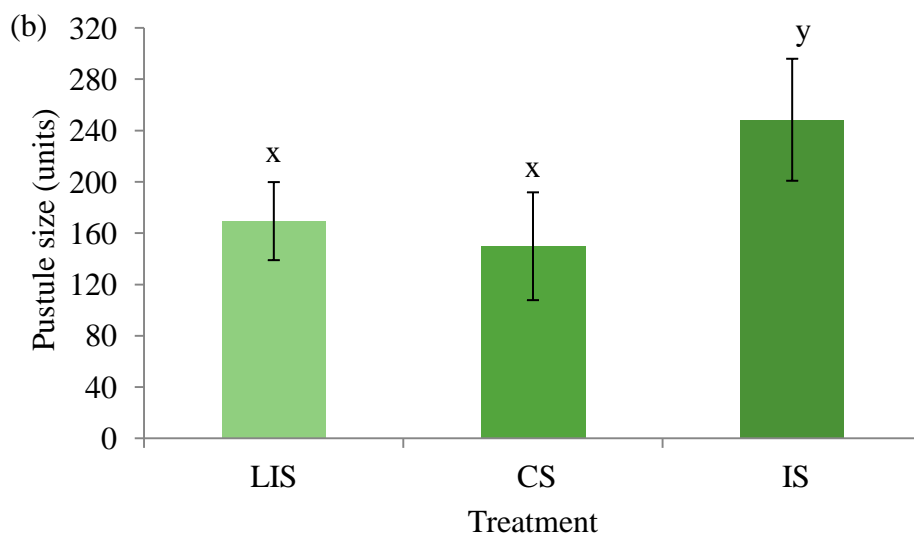
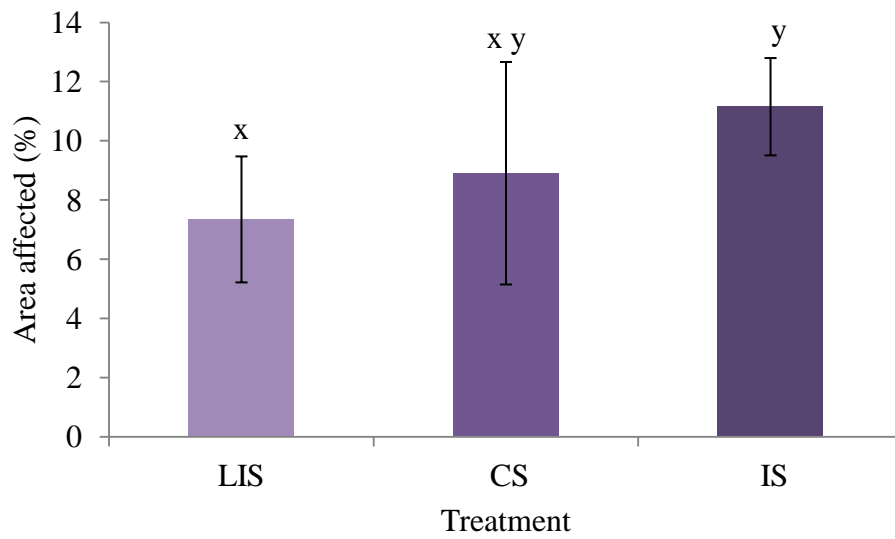


Figure 4.4: Leaf rust development in infected susceptible wheat seedlings after exposure to volatiles emitted by susceptible seedlings. (a) Percentage area affected and (b) relative pustule size were calculated from photographs taken at 10 dpi of susceptible wheat seedling leaves. Measurements were determined for later infected susceptible (LIS), control susceptible (CS) and infected susceptible (IS) seedlings exposed to either IS, MS or typical glasshouse volatile emissions respectively. Treatment means with different letters differ significantly ($P < 0.05$) as determined by a Tukey's multiple comparison test. $n = 10$ leaves.

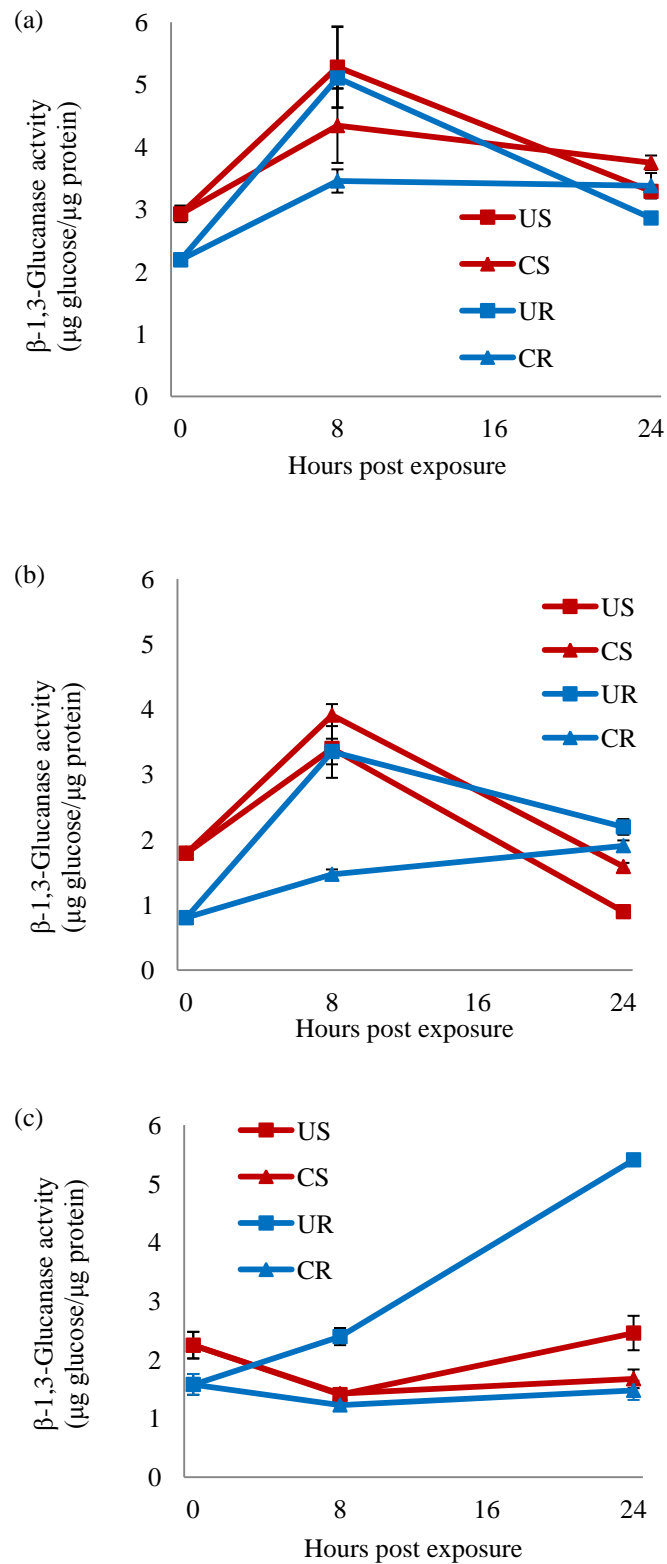


Figure 4.5: β -1,3-Glucanase activity in wheat seedlings exposed to volatiles emitted by resistant seedlings. Uninfected resistant (UR) and susceptible (US) plants were exposed to infected resistant plants and control resistant (CR) and susceptible (CS) seedlings to mock infected resistant plants. The experiment was conducted in triplicate (a-c). $n = 3$ enzyme activity measurements.

In summary, resistant seedlings exposed to volatiles emitted by infected resistant seedlings showed a much stronger response compared to the susceptible seedlings, while a weaker induced response was also visible in the control seedlings.

For the experiment using susceptible seedlings as volatile source, all replicates showed similar β -1,3-glucanase activity profiles (figure 4.6). Enzyme activity increased from 0 hpe to a maximum at 8 hpe with a subsequent decrease towards 24 hpe. β -1,3-Glucanase activity in all the UR plants was induced at 8 hpe with the activity in the CR plants remaining constant or decreasing. On average, this increase in activity in the UR plants was twofold in all three replicates. β -1,3-glucanase activity followed the same trend in the exposed susceptible plants with an average 1.9 fold increase in the three US replicates. Activity in two of the CS replicates increased 1.4 fold while the third showed a decrease (figure 4.6).

In summary, UR and US plants showed a similar 2 fold increase in β -1,3-glucanase activity after exposure to volatiles emitted by IS plants, which was only slightly higher compared to the controls (figure 4.6). This activity was however about half that of the UR and US plants exposed to volatiles emitted by the IR plants (figure 4.5).

4.4 *PR2* gene expression analysis of volatile exposed plants

Northern blot analysis was initially attempted to determine *PR2* gene expression, but the mRNA levels of the tested genes were too low to detect. Despite its limitations, RT-PCR was then decided upon to confirm any induction of *PR2* expression in volatile exposed seedlings by using the constitutively expressed *actin* gene as reference. A representative agarose gel result for all three replicates is shown in figures 4.7 and 4.8.

PR2 gene expression is induced at 8 hpe in UR and US seedlings after exposure to volatiles emitted by IR seedlings (figure 4.7). A subsequent decrease in expression was seen at 24 hpe. Expression in the control treatments (CR and CS) showed either no induction or a decrease in *PR2* expression over the tested time intervals. Figure 4.8 shows *PR2* expression in the seedlings that were exposed to volatiles emitted by susceptible wheat. *PR2* expression is induced in the UR and CR plants at 8 hpe with a subsequent decrease in expression at 24 hpe (figure 4.8a). US and CS in turn showed no induction of gene expression.

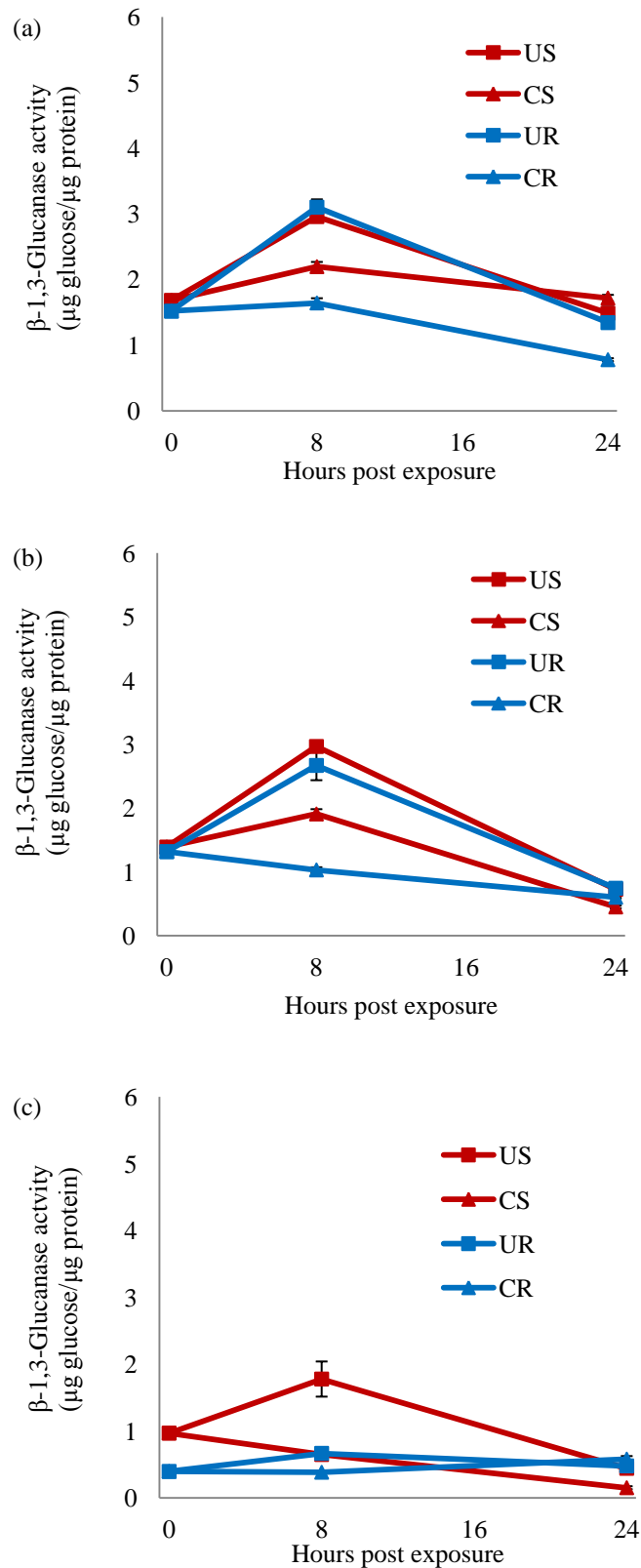


Figure 4.6: β -1,3-Glucanase activity in wheat seedlings exposed to volatiles emitted by susceptible seedlings. Uninfected resistant (UR) and susceptible (US) plants were exposed to infected susceptible plants and control resistant (CR) and susceptible (CS) seedlings to mock infected susceptible plants. The experiment was conducted in triplicate (a-c). $n = 3$ enzyme activity measurements.

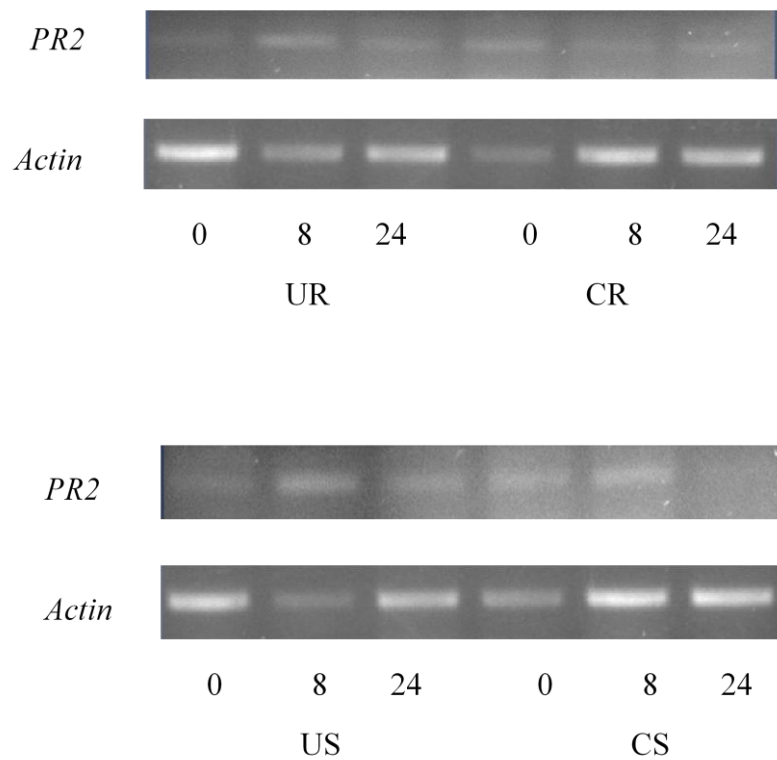


Figure 4.7: Relative gene expression of *PR2* in wheat seedlings exposed to volatiles emitted by resistant seedlings. Uninfected resistant (UR), control resistant (CR), uninfected susceptible (US) and control susceptible (CS) seedlings were exposed to either infected resistant (IR) or mock infected resistant (MR) volatile emissions.

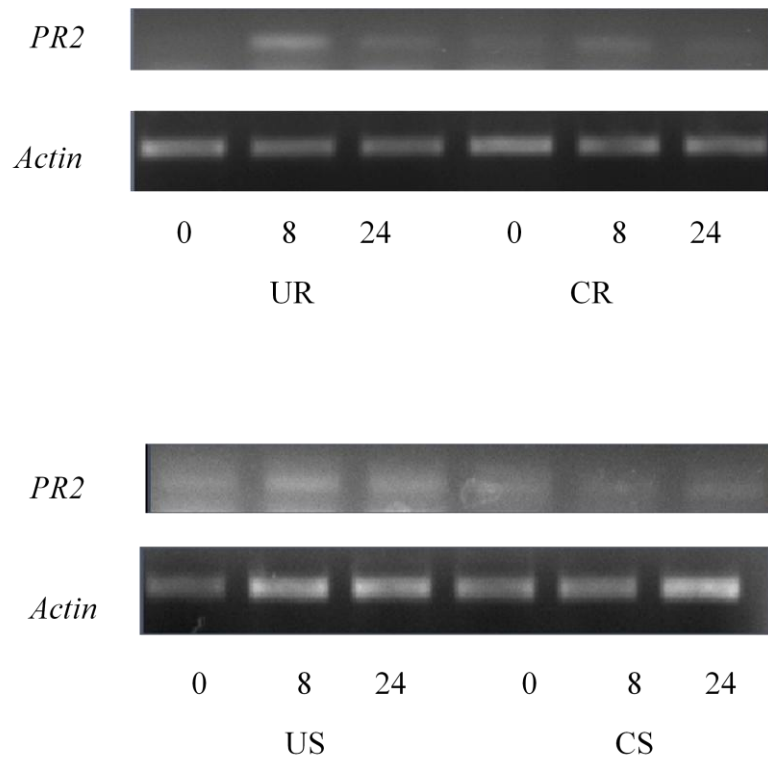


Figure 4.8: Relative gene expression of *PR2* in wheat seedlings exposed to volatiles emitted by susceptible seedlings. Uninfected resistant (UR), control resistant (CR), uninfected susceptible (US) and control susceptible (CS) seedlings were exposed to either infected susceptible (IS) or mock infected susceptible (MS) volatile emissions.

4.5 Analysis of volatiles from seedlings

To capture and analyse volatiles emitted by infected seedlings, a number of methods was investigated and optimised. Initially it was attempted to trap volatiles by using a porous polymer while air was circulated through the closed system. The setup however proved inadequate to trap volatiles (results not shown), suggesting that the volatiles may be emitted at low levels. For this reason SPME was decided upon and proved to be more efficient.

The GC/MS profiles for IR and MR seedlings are shown in figure 4.9, while the IS and MS profiles are shown in figure 4.10. Since the analysis was not quantitative, the relative percentage of compounds cannot be compared between different analyses but only within a given profile. The most intense peaks were identified using MS and compounds were compared against a database of known plant volatiles. Compounds that were products of column bleed or contaminants are for the most part not reported. Some contaminants such as toluene, *p*-xylene and diethyl phthalate are indicated among the identified compounds (tables 4.1 and 4.2).

When looking at the identified volatiles from resistant seedlings (table 4.1) a marked difference in volatile composition between MR and IR seedlings can be observed. IR had a number of volatiles unique to the treatment. Some unique compounds included fatty acid derivatives (decane, undecane, and 2-ethylhexanol), benzenoids (styrene, benzaldehyde and 1,2,4-trimethyl-benzene) and a terpenoid (ocimene). Only two fatty acid derived alkanes (3-methyl-tridecane and pentadecane) were unique to MR.

Volatiles from susceptible seedlings (table 4.2) showed less difference between the respective treatments. IS had only three unique volatiles that are all fatty acid derivatives namely (*Z*)-2-pentenyl acetate, 3-cyclohexenyl acetate and undecane. MS in turn had only one unique volatile, namely octen-3-ol.

Comparing all treatments of resistant and susceptible seedlings, a clear difference was observed even though the lines (Thatcher and Thatcher+*Lr9*) are near-isogenic. Volatiles from the fatty acid derivate, benzenoid and terpenoid classes were detected in the infected resistant seedlings. Many of these volatiles were unique to the IR treatment and were not observed in any other treatment. In the susceptible line, only fatty acid derivatives were observed and GLV's seemed to be dominant. However a number of these volatiles are unique to the susceptible treatments (IS and MS).

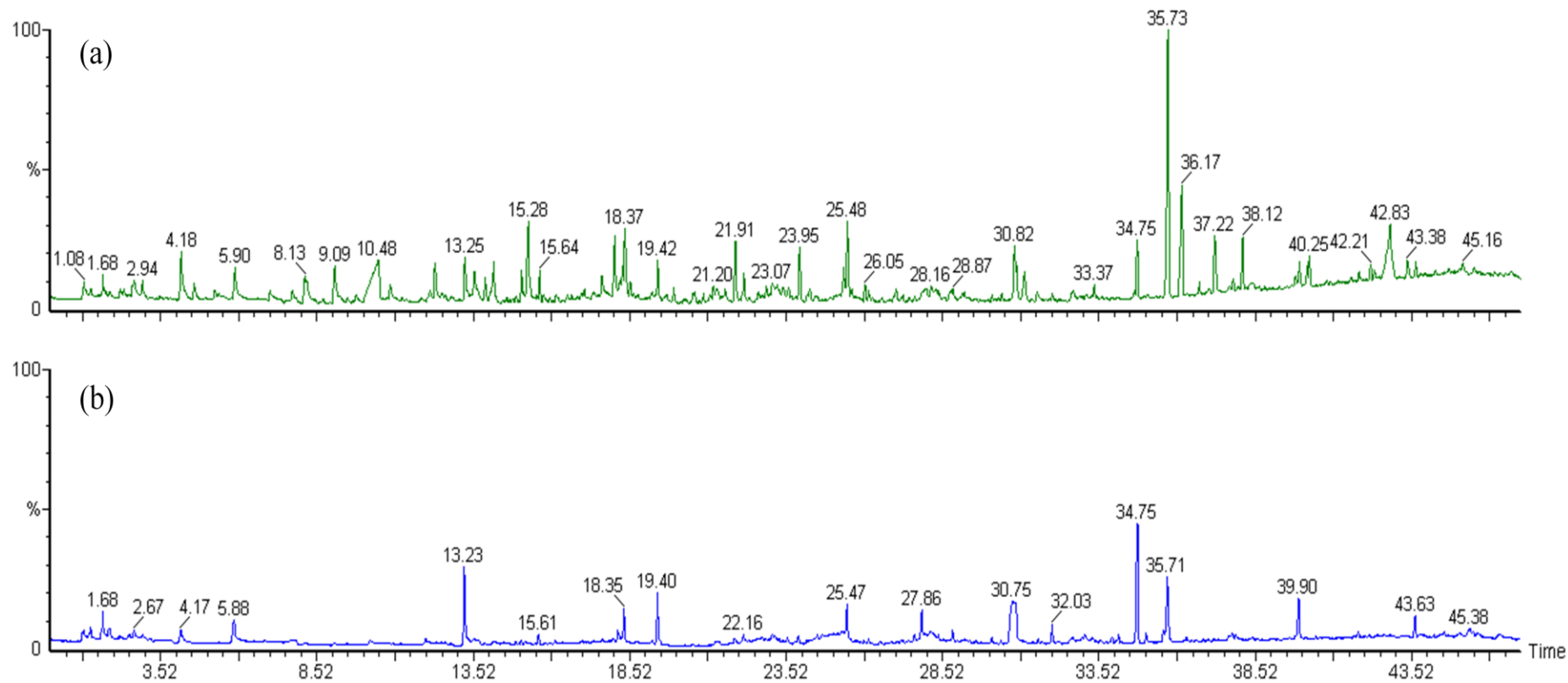


Figure 4.9: Gas chromatography profiles for compounds captured by solid phase micro-extraction from infected resistant wheat seedlings five days post infection. Profiles are shown for (a) infected resistant (IR) and (b) mock infected resistant (MR) seedlings.

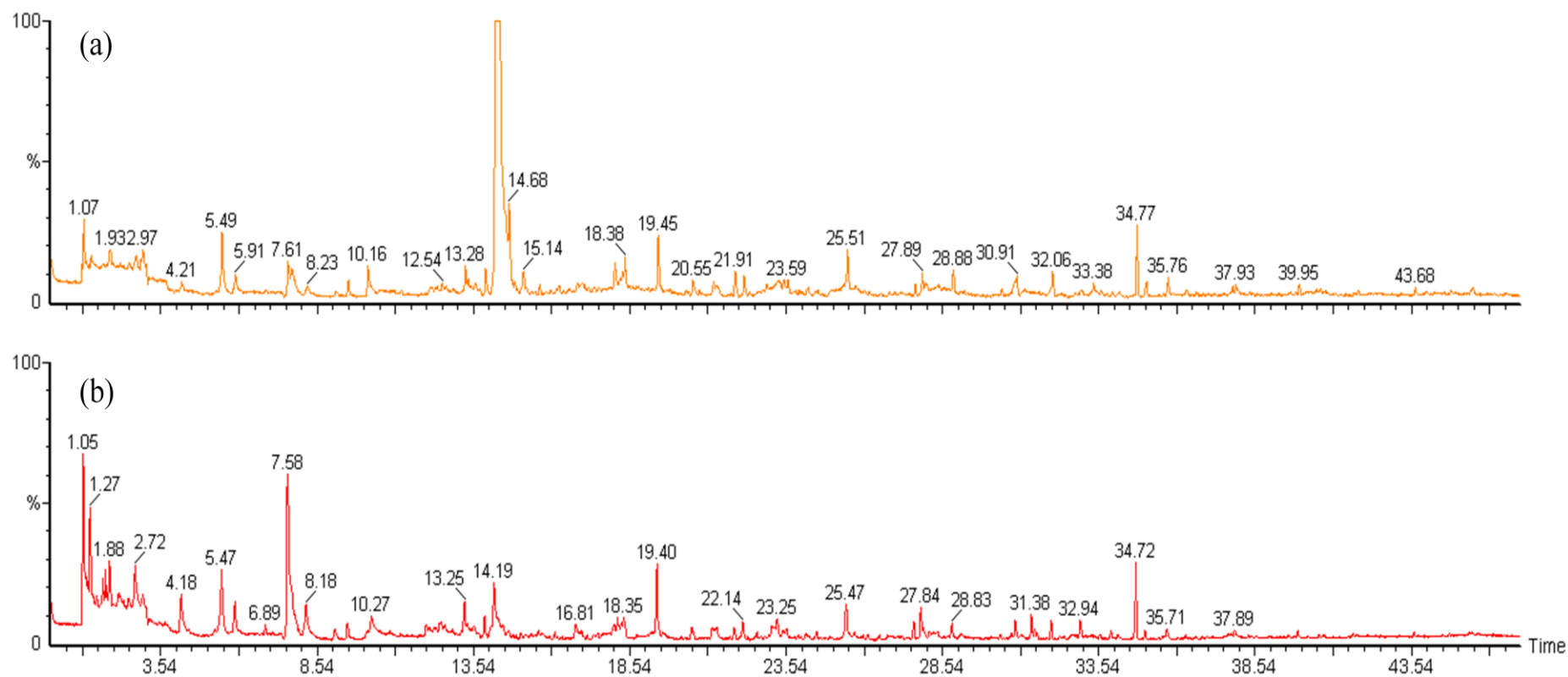


Figure 4.10: Gas chromatography profiles for compounds captured by solid phase micro-extraction from infected susceptible wheat seedlings five days post infection. Profiles are shown for (a) infected susceptible (IS) and (b) mock infected susceptible (MS) seedlings.

Table 4.1: Comparison of selected volatile compounds identified from mock infected resistant (MR) and infected resistant (IR) seedlings five days post infection. Identification was done by matching mass spectra against reference spectra on a database. Retention time indicated corresponds to a peak on the gas chromatography profile. Certain compounds were not detected (ND) for a given treatment.

Compound	Retention Time	
	MR	IR
Toluene	4.151	4.180
2,4-Pentanedione	ND	4.591
<i>p</i> -Xylene	8.130	8.130
Styrene	ND	9.090
Butyrolactone	ND	10.480
Benzaldehyde	ND	12.305
1,2,4-Trimethyl-benzene	ND	13.580
Decane	ND	13.895
Ocimene	ND	14.179
D-Limonene	15.075	15.060
2-Ethylhexanol	ND	15.296
Undecane	ND	18.04
2-Ethylhexyl acetate	ND	19.924
3-Methyl-tridecane	27.858	ND
Dodecane	ND	21.910
Decanal	ND	22.160
Pentadecane	32.030	ND
Diethyl Phthalate	34.722	34.750

Table 4.2: Comparison of selected volatile compounds identified from mock infected susceptible (MS) and infected susceptible (IS) seedlings five days post infection. Identification was done by matching mass spectra against reference spectra on a database. Retention time indicated corresponds to a peak on the gas chromatography profile. Certain compounds were not detected (ND) for a given treatment.

Compound	Retention Time	
	MS	IS
(<i>E,E</i>)-2,4-Hexadienal	2.719	2.954
Toluene	4.180	4.214
(<i>E</i>)-2-Hexenal	7.680	7.610
(<i>E</i>)-2-Hexenol	8.180	8.230
<i>p</i> -Xylene	9.090	ND
Nonane	9.503	9.550
(<i>Z</i>)-2-Pentenyl acetate	ND	10.180
Decane	13.895	13.942
(<i>Z</i>)-3-Hexenyl acetate	14.190	14.289
3-Cyclohexenyl acetate	ND	15.140
Octen-3-ol	16.810	ND
Undecane	ND	18.067
Decanal	22.140	22.207
3-Methyl-tridecane	27.840	27.905
Tetradecane	28.830	28.865
Pentadecane	32.014	32.060
Diethyl Phthalate	34.720	34.770

Chapter 5: Discussion

The perception and induced defence response of plants against herbivores and pathogens are dissimilar and it is expected that the emitted volatiles will also differ. A study by Cardoza *et al.* (2002) compared volatiles emitted by peanut plants after white mould infection and beet armyworm infestation respectively. The number of different volatiles and relative quantities of these compounds were much lower upon white mould infection. Infection with white mould does however cause the emission of a number of unique volatile compounds.

In contrast a number of volatiles seem to overlap quantitatively when comparing *Fusarium* spp. infection and *Oulema* spp. infestation in wheat (Piesik *et al.*, 2011a). It was argued that fungal infection causes damage over a long time scale while herbivores cause severe tissue damage. Whatever the nature of volatile emissions the role of these compounds in interplant signalling after herbivory or mechanical damage is well established (Heil and Karban, 2010). In this review of interplant signalling only a limited number of examples are given considering pathogenic infection. Recently interplant signalling however has been proven following *Fusarium* infection of barley, oat and wheat (Wenda-Piesik *et al.*, 2010) and maize (Piesik *et al.*, 2011b).

Appelgryn (2007) observed a putative signalling event in wheat infected with leaf rust, using both resistant (Thatcher+*Lr34*) and susceptible (Thatcher) lines. In his study, infected resistant and susceptible wheat seedlings were respectively placed in an enclosed chamber together with uninfected (both resistant and susceptible) wheat seedlings. The general trend showed an induction in *PR2*, *PR5* and *TaHlp01* gene expression in the exposed uninfected seedlings. β -1,3-Glucanase activity also increased in uninfected wheat seedlings for certain Thatcher line combinations. The plants had no physical contact except through the air in the enclosed chambers, thus signalling via released volatiles was the only probable explanation. It was noted that the phenomena was more significant between infected and uninfected plants of the same Thatcher line.

The aim of the current project was to firstly confirm the putative signalling event between leaf rust infected and uninfected wheat. The hypothesis was that wheat seedlings infected with *P. triticina* emit volatile compounds that may diffuse to nearby uninfected wheat seedlings and correspondingly induce a defence response in these plants. The induced defence in the uninfected wheat seedlings may then grant a competitive advantage during any subsequent infection. The second aim was to investigate whether any susceptible/resistant

interactions are present. Finally, the types of volatiles emitted by the two wheat lines upon *P. triticina* infection were identified and a putative important volatile signal identified.

Initially, the study aimed to verify and elaborate on the research of Appelgryn (2007) by addressing relevant criticism. One criticism that may be brought forward against the previous work involves the experimental setup itself. Enclosing plants in a chamber may allow the accumulation of volatiles to unnatural levels (Dicke *et al.*, 2003; Paschold *et al.*, 2006) and by extension lead to a response in uninfected plants never observed under field conditions. The newly designed experimental system used in the current study does not allow volatiles to accumulate (figures 3.1 and 3.2). The advantage of the system was that it did allow the directional control of airflow between infected and uninfected plants. It also allowed the simultaneous use of an uninfected control under the exact same conditions without cross contamination of volatiles.

Experimentation with the goal to prove interplant signalling must take other factors into consideration. The possibility of molecules diffusing to neighbouring plants via the soil and inducing defence mechanisms has been proven in lima bean plants (Dicke and Dijkman, 2001). In broad bean, signal molecules may also be transmitted upon infestation by aphids, mediating a tritropic interaction in neighbouring plants. The signal molecule(s) could be transmitted via the soil or hydroponic system and not exclusively via air currents (Guerrieri *et al.*, 2002). Any study in possible volatile signalling must thus eliminate volatile transfer by any means other than by the atmosphere itself if a deduction is to hold true. The current system was designed in such a way that during the 24 h volatile exposure period there was no contact via soil or water between seedlings (figures 3.2 and 3.3). Therefore if any volatile signal was transferred between the infected and uninfected plants it was exclusively via the directional air current in the system.

Throughout the entire experimentation procedure, infected and uninfected plants were kept separately in the glasshouse except during the 24 h volatile exposure period. Spore transfer could be excluded during this 24 h period as spore formation on the infected plants had not yet occurred at 5 dpi, but only occurred at 7-10 dpi (Bolton *et al.*, 2008b). The conditions during the 24 h period were also not favourable for the accidental inoculation of uninfected wheat seedlings. The experimental setup excluded any direct effect that volatiles might have on spore germination as inoculation of exposed plants only followed after the volatile source plants were removed from the system. Leaf rust urediospores inoculated onto exposed plants

were therefore only exposed to typical glasshouse volatiles. Any response in the experimental seedlings exposed to volatiles must therefore be due to the perception of such a compound by the exposed plants.

During the initial phase of the study 1, 5 and 10 dpi wheat seedlings were used as volatile sources. Seedlings 1 and 10 dpi did not seem to induce a defence response in uninfected seedlings (results not shown) and the 5 dpi seedlings were subsequently used. The choice of 5 dpi seedlings as volatile source was supported by the observation by Toome *et al.* (2010) of an increase in volatile emissions 6 dpi in willow trees infected with *Melampsora epitea*. Toome *et al.* (2010) also observed a later induction of volatiles at 12 dpi and it is possible that in wheat volatiles may increase again after 10 dpi.

At phenotypical level, it was observed that previous exposure to the emitted volatiles reduced the effects of a subsequent leaf rust infection (figures 4.3 and 4.4). The effect was more pronounced when the infected resistant line (Thatcher+*Lr9*) was used as volatile source (figure 4.3). Initially this clearly indicated a benefit gained by the susceptible line simply by being in contact with infected seedlings via air currents. Appelgryn(2007) speculated that interplant signalling may be better between similar wheat lines but the phenotypical data of the current study proved otherwise. The resistant line was superior to the susceptible line in granting the seedlings some minor resistance, the nature of which is not deducible. Exposure to IR seedlings significantly decreased the percentage area affected in LIS relative to CS exposed to MR. But after the same exposure the pustule size did not differ significantly between LIS and CS. It may be argued that IR exposure decreased the number of infection occurrences but did not inhibit the disease progression. This effect was not seen in the treatments that were exposed to susceptible seedlings (IS and MS).

It should therefore be noted that when considering the phenotypical data, no deduction could be made whether the differences were attributed to an induction of defence or through the priming of the defence response. If volatiles act as priming agents, a secondary inducing agent such as a pathogen infection is still needed to induce the defence response (Conrath *et al.*, 2006). The response however may be greater and/or earlier in the primed seedlings.

In a study by Yi *et al.* (2009) uninfected lima bean plants were exposed to volatiles emitted by plants where SAR was artificially induced by benzothiadiazole. The exposed plants were then subsequently infected with *P. syringae*. In the infected plants previously exposed to volatiles from SAR induced plants a higher level of *PR2* gene expression was observed

compared to those exposed to volatiles from untreated plants. In contrast volatiles may also induce defence without any secondary inducing agent being present (Kishimoto *et al.*, 2005; Kishimoto *et al.*, 2007). Both these mechanisms may therefore equip the volatile exposed seedlings to combat the subsequent leaf rust infection. The other analyses that were conducted and discussed hereafter only considers a direct induction of defence but future research must consider the existence of a primed state.

When considering the β -1,3-glucanase activity it should firstly be noted that the levels were much lower than previously reported (Cawood *et al.*, 2010). During the current study, a total protein extract from leaf tissue was used for the determination of β -1,3-glucanase activity, while previous studies used apoplast protein extracts, a fraction with a much higher β -1,3-glucanase activity per protein extracted. This may also be the reason why chitinase and peroxidase activity could not be measured. This observation may be compounded by the fact that the experimental system did not allow for the accumulation of volatiles. Previous results have indicated that a high volatile concentration induces a stronger defence response, with methyl jasmonate greatly increasing chitinase and peroxidase activity but only when supplied as a foliar spray or seed treatment (Haggag and Abd-El-Kareem, 2009).

In general, all seedlings exposed to volatiles showed an induction of β -1,3-glucanase activity reaching maximum activity at 8 hpe (figures 4.5 and 4.6). The pattern of the induced β -1,3-glucanase activity by volatile exposure differs from that seen during a typical rust infection. Upon leaf rust infection, β -1,3-glucanase activity is induced in resistant and susceptible wheat seedlings and as the disease progresses still increases after 24 hpe (Anguelova-Merhar *et al.*, 2001). Volatile exposure in contrast only leads to a brief period of induced β -1,3-glucanase activity. In all cases, the induced enzyme activity in the UR/CR seedlings was more pronounced than that of the US/CS seedlings, regardless of the volatile source.

Wheat has a number of genes in the *PR2* family that encode β -1,3-glucanases which all contribute to enzymatic activity. It should be noted that these enzymes may be differentially induced in response to different pathogens or developmental stages (Higa-Nishiyama *et al.*, 2006). In this project the induced enzyme activity was found using the pool of β -1,3-glucanases in a total protein extract. The activated enzyme level is however not an indication of the expression of the different *PR2* genes that contribute to the total enzyme activity.

A total RNA extract from leaf tissue was used to track *PR2* gene expression relative to the constitutively expressed *actin* gene. *PR2* gene expression was used as another marker of an

induced defence response and for comparison with β -1,3-glucanase activity. For all three replicates, resistant seedlings exposed to volatiles emitted by infected resistant seedlings showed an induction of *PR2* expression at 8 hpe (figure 4.7). In turn, only one of the three replicates showed induction of *PR2* expression in susceptible seedlings exposed to infected resistant seedlings. Seemingly, the expression of *PR2* was less likely to be induced in exposed susceptible seedlings. When infected susceptible seedlings served as volatile source, all replicates of exposed resistant seedlings showed an induction of *PR2* at 8 hpe (figure 4.8). In turn none of the exposed susceptible seedlings showed an induction of *PR2* at any time interval. The observation of induced β -1,3-glucanase activity and *PR2* expression supports the deduction that resistant seedlings have an intrinsic ability to induce a defence response upon perception of volatile signals and more so than susceptible seedlings.

In most cases the increased β -1,3-glucanase activity compared well with induced gene expression. β -1,3-Glucanase (on gene and enzymatic levels) was induced rapidly at 8 hpe but thereafter decreased towards 24 hpe. The observation raises the question whether β -1,3-glucanase contributed to the defence response seen on phenotypic level in susceptible wheat if the enzyme activity has already decreased at 24 hpe. The gene expression level may well be induced before 8 hpe but also decreases quickly thereafter. Gene expression is not always a direct indication of enzyme activity and it is probable that once the subsequent infection occurs enzyme activity will again increase. However, the brief induction of β -1,3-glucanase on genetic and active enzyme level still indicated that a volatile signal was perceived and a defence reaction induced in the absence of a pathogen. The complexity of this response and the duration thereof must however be further investigated and cannot be speculated upon.

When considering all the data, it was clear that the CR and CS seedlings in some cases induced a defence response although at much lower levels than the experimental samples. Two possible explanations can be brought forward to explain this observation. Firstly, transfer of seedlings to the volatile chamber from the glasshouse may have placed the seedlings under a brief stress period that may have induced the defence response. Secondly, it may be true that the infection procedure may place the seedlings under brief abiotic stress, thereby inducing a possible volatile emission event whether the infection was true or mock. The contribution of the kerosene oil used during infection must also be considered. The strength of the induced defence response was however always less intense if mock infected seedlings were the source of volatiles. Infection therefore contributed to the signalling event in addition to any abiotic effects that may have been present.

The volatile exposure during the current study was for only 24 h and not over an extended period of time. Further studies may have to include a longer volatile exposure time, more time intervals and most importantly a greater number of defence parameters. However, during all experimentation, IR wheat seedlings were considered the best inducer of defences in nearby uninfected seedlings regardless of the line. Therefore a novel volatile compound(s) present in IR wheat seedlings, but absent in IS, MR or MS seedlings, could be crucial for the stronger induced defence response.

The uninfected plants (UR, CR, US and CS) were exposed to all volatiles present in the experimental system. These volatiles may be derived from the seedlings, the pathogen, the soil, the kerosene used during infection and any other minor contaminants. The contribution of each of these sources to the total volatile mix might need further investigation in future so as to define the role of each if they are indeed involved. The total mix of volatiles was however captured and analyzed with GC-MS to identify putative volatile signals. Toluene, *p*-xylene and decane are derived from kerosene oil (Pandit *et al.*, 2001) and can therefore be considered possible contaminants and be excluded from the analyses. All the other identified volatiles are therefore either of plant or fungal origin.

When comparing the composition of the IR and MR volatile bouquets (table 4.1), a large number of volatiles were unique to the IR treatment. Ocimene, ethylhexanol and decanal were observed in IR but not in the MR treatment. The volatile blend emitted by lima bean plants contained ocimene, ethylhexanol and decanal amongst others (Heil and Bueno, 2007). This mixture of volatiles primed the defence response against leaf beetle infestation in neighbouring lima bean plants. However, ethylhexanol and decanal could not be linked to induced defence against fungal pathogens.

Butyrolactone emissions have been shown to be induced by the infection of wheat grains by certain *Fusarium* spp. (Eifler *et al.*, 2011). Butyrolactone along with styrene, undecane, dodecane and pentadecane have all been identified in natural occurring wheat grains (Buško *et al.*, 2010) but were not directly linked to an induced defence response against fungal pathogens. Styrene is emitted by the fungal pathogen *Fusarium oxysporum* Schldl. (Beck *et al.*, 2008). The emission of styrene from IR may therefore be attributed to the leaf rust fungus itself. Other unique volatiles present in the IR (2,4-pentanedione, 1,2,4-trimethyl-benzene and ethylhexyl acetate) and MR (methyl-tridecane) treatments could not be directly linked to induced defence against fungal pathogens.

Benzaldehyde was unique to the IR treatment and has previously been found in young wheat leaves (Shibamoto *et al.*, 2007). Benzaldehyde however seem to be emitted generally by wheat during aphid infestation, aphid associated enzyme (i.e. cellulase) treatments and even after a water treatment (Liu *et al.*, 2009). Benzaldehyde could however not be linked to induced defence against fungal pathogens.

As stated above ocimene was another volatile that was unique to IR but absent in MR. Previous research has indicated the importance of this compound in plant defence. The emission of ocimene was induced after infection of willow trees by *M. epitea* at 6 dpi (Toome *et al.*, 2010). Ocimene also induces a number of defence related genes in *Arabidopsis*, thereby improving the defence against a subsequent *B. cinerea* infection (Kishimoto *et al.*, 2005). Ocimene also induces lignification, accumulation of the toxin camalexin and *PR3* expression in *Arabidopsis* (Kishimoto *et al.*, 2006). However, the ocimene response was independent of *PR1* and *PR2* gene expression induction in *Arabidopsis* (Kishimoto *et al.*, 2005; Kishimoto *et al.*, 2006).

Volatiles from IS and MS (table 4.2) showed little differences with both emitting a number of GLV's (table 4.2). (*Z*)-2-Pentenyl acetate and (*Z*)-3-cyclohexenyl acetate, which were observed in IS but in the MS treatment, could not be linked to the induction of defences against fungal pathogens and require further investigation.

Volatiles such as (*E*)-2-hexenal, (*E*)-2-hexenol and (*Z*)-3-hexenyl acetate are emitted upon infection of wheat with a blend of *Fusarium* spp. (Piesik *et al.*, 2012). (*Z*)-3-hexenyl acetate has been shown to induce jasmonic acid accumulation (Engelberth *et al.*, 2004) and genes coding for downstream lipoxygenase pathway proteins (Engelberth *et al.*, 2007). (*Z*)-3-Hexenyl acetate also primes a number of defence related genes in poplar trees (Frost *et al.*, 2008). Whether (*Z*)-3-hexenyl acetate can induce *PR2* expression requires further investigation. (*E*)-2-Hexenal induces a number defence related genes in *Arabidopsis* and thereby improves the defence against a subsequent *B. cinerea* infection, but the response was shown to be independent of *PR2* gene expression induction (Kishimoto *et al.*, 2005). (*E*)-2-Hexenol induces a number of genes in maize including *lox*, *pal* and *maize protein inhibitor* (Frag *et al.*, 2005)

The GLV's observed in both IS and MS most probably contributed to the defence response seen in both experimental and control plants. The volatile octen-3-ol which was unique to the MS treatment may also have contributed to the induction observed in control samples.

Octen-3-ol has been shown to induce an antifungal *defensin* gene and *PR3* in *Arabidopsis* but does not induce *PR2* (Kishimoto *et al.*, 2007).

All resistant and susceptible treatments were qualitatively different even though the lines utilized are near-isogenic. Resistant (Thatcher+*Lr9*) seedlings emitted volatiles from various classes, while susceptible (Thatcher) seedlings emitted mainly GLV's. During the analysis a large number of GLV's in the susceptible treatments may have obscured rarer volatiles that were seen in resistant treatments. The contribution of the resistance genes and the resistance response to volatile profiles must be investigated in future. Improving capturing and analysis of volatile compounds from wheat may resolve some of the apparent differences.

In comparison, in both resistant and susceptible peach plants a quick increase of GLV's and benzaldehyde was induced by mechanical damage and aphid infestation (Staudt *et al.*, 2010). In the current study GLV's were unique to susceptible wheat and benzaldehyde only to IR wheat. Furthermore resistant peach cultivars also induced various terpenoid volatiles upon aphid infestation with one being ocimene (Staudt *et al.*, 2010). Likewise ocimene was an important volatile that was present in IR wheat and absent in MR, IS and MS treatments.

The defence markers used in the study (phenotype, gene expression and enzyme activity) indicated that volatiles from the IR seedlings caused a more pronounced defence response in uninfected seedlings. Except for ocimene, a limited number of volatiles that were unique to IR wheat have been implicated in defence against pathogens. It may be speculated that resistant wheat causes induced defence via ocimene or other volatiles that have not been described as functional. Susceptible wheat in turn causes induced defence via GLV's; a group of volatiles that have been thoroughly described as functional.

In conclusion it appears that when uninfected wheat seedlings were exposed to volatiles from *P. triticina* infected seedlings, a transient induction of defence was observed. This defence response was marked by increased *PR2* expression and β -1,3-glucanase activity. The subsequent infection of exposed susceptible (Thatcher) seedlings also indicated an induced defence response. However, resistant (Thatcher+*Lr9*) wheat may have the intrinsic ability to induce a stronger defence response when volatiles are perceived. *PR2* gene expression and β -1,3-glucanase activity as investigated here are merely markers for the defence response, and the response surely is far more complex. Future research into the induced defence by volatiles must follow scientific trends and look at the bigger picture of the cellular response.

Proteomics and transcriptomics will allow the description of many other defence components simultaneously.

From this study, it is proposed that volatiles such as ocimene from infected Thatcher+*Lr9* and GLV's from infected Thatcher may be the best candidates for putative signals between wheat seedlings. It is apparent that several of the other identified volatiles need further investigation as these compounds may act as priming/inducing agents in wheat but have not been described as such before. Research should track volatile emissions in parallel with the development of *P. triticina* on wheat over a number of days in order to identify other putative signals.

Another avenue worth investigating is the direct effect the emitted volatiles may have on the germination of leaf rust spores thereby identifying new rust control agents. A number of volatiles identified in our study have been implied to affect pathogenic development. Ocimene reduces the length and penetration of *B. cinerea* hyphae on *Arabidopsis* (Kishimoto *et al.*, 2006). (*Z*)-3-Hexenyl acetate inhibits the growth of *S. rolfsii* on peanut plants (Cardoza *et al.*, 2002) and octen-3-ol reduces lesion size of *B. cinerea* on *Arabidopsis* (Kishimoto *et al.*, 2007). The rate of germination and hyphal length of *B. cinerea* was greatly reduced when exposed to a mixture of hexanal isomers including (*E*)-2-hexenal (Kishimoto *et al.*, 2008). Decanal however promotes the growth of *P. graminis* on wheat (Mendgen *et al.*, 2006).

The research presented here confirms the hypothesis that interplant signalling occurs between leaf rust infected wheat and uninfected wheat. This is confirmed under controlled glasshouse conditions and can be attributed to number of volatile compounds emitted by infected wheat. Whether or not interplant signalling occurs frequently under field conditions must still be confirmed. Investigating the phenomenon under controlled conditions may however pave the way towards the identification of new volatile elicitors of defence. Such volatile elicitors can be easily applied but will be naturally degraded in the atmosphere. Genetic manipulation may also allow plants to emit volatiles at far greater quantities and allow noticeable effects under field conditions. These effects will also be well-defined and predictable for agricultural systems that normally utilize the same species and cultivar in the field.

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Summary

Plants emit a vast array of volatile organic compounds to which surrounding plants can respond. Recent research suggested volatile signalling between leaf rust (*Puccinia triticina* Erikss.) infected and uninfected wheat (*Triticum aestivum* L.). The current project was undertaken to further investigate these putative events. Uninfected wheat was exposed to volatiles emitted by leaf rust infected wheat in a continual air flow system. Different combinations of resistant (Thatcher+*Lr9*) and susceptible (Thatcher) wheat lines were used for the volatile exposure experiments. When susceptible seedlings were exposed to volatiles from either resistant or susceptible plants there was a significant decrease in percentage leaf area infected and pustule size after a subsequent infection with leaf rust. The volatile exposure was also linked with an induced defence response as confirmed with increased enzyme activity and gene expression. Generally, an induction of β -1,3-glucanase activity was observed at 8 hours post exposure in exposed resistant and susceptible seedlings regardless of the infected line that released the volatiles. *Pathogen-related protein 2* gene expression was also induced at 8 hours post exposure in both lines exposed to volatiles released by infected resistant seedlings but not in those exposed to infected susceptible seedling volatiles. This induction of the defence response could only be attributed to volatiles emitted by infected wheat seedlings. Emitted volatiles were captured by solid phase micro-extraction and classified by gas chromatography mass spectrometry. Infected resistant seedlings released a number of unique volatiles including ocimene that was not observed in both the mock infected resistant or infected and mock infected susceptible seedlings. Fewer unique volatiles were observed in the infected susceptible seedlings compared to the mock infected susceptible seedlings with green leaf volatiles being common in both treatments. Some of the identified volatiles were previously linked to the defence response in plants.

Opsomming

Plante stel 'n wye verskeidenheid van vlugtige organiese verbindings vry waarop omliggende plante kan reageer. Onlangse navorsing het getoon dat seinoordrag tussen blaarroes (*Puccinia triticina* Erikss.) geïnfekteerde en ongeïnfekteerde koring (*Triticum aestivum* L.) deur middel van vlugtige verbindings kan geskied. Die huidige projek was onderneem om hierdie veronderstelde kommunikasie verder te ondersoek. Ongeïnfekteerde koring was aan vlugtige verbindings blootgestel wat deur blaarroes geïnfekteerde koring vrygestel was binne in 'n aaneenlopende lugvloei-sisteem. Verskillende kombinasies van weerstandbiedende (Thatcher+*Lr9*) en vatbare (Thatcher) koringlyne is gebruik. Wanneer vatbare saailinge aan vlugtige verbindings vanaf weerstandbiedende of vatbare plante blootgestel was, was 'n afname in persentasie area geïnfekteer en puisie grootte tydens 'n daaropvolgende infeksie waargeneem. Die blootstelling aan vlugtige verbindings was ook gekoppel aan geïnduseerde verdediging soos gekenmerk deur 'n toename in ensiemaktiwiteit en geenuiting. 'n Induksie van β -1,3-glukanase aktiwiteit was by 8 ure na blootstelling in weerstandbiedende en vatbare saailinge gesien ongeag die lyn vanwaar die vlugtige verbindings afkomstig was. *Patogeen-geassosieerde proteïen 2* geenuiting was ook geïnduseer 8 ure na blootstelling in beide lyne blootgestel aan geïnfekteerde weerstandbiedende saailinge, maar nie in dié wat blootgestel was aan vatbare saailinge nie. Die induksie van 'n verdedigingsrespons kan net toegeskryf word aan vlugtige verbindings afkomstig vanaf geïnfekteerde saailinge. Vlugtige verbindings was deur soliede fase mikroekstraksie vasgevang en geklassifiseer met behulp van gaskromatografie massaspektrometrie. Geïnfekteerde weerstandbiedende saailinge het 'n aantal unieke vlugtige verbindings vrygestel, insluitend ocimene, wat nie in die ongeïnfekteerde weerstandbiedende, geïnfekteerde vatbare of ongeïnfekteerde vatbare saailinge teenwoordig was nie. Slegs 'n klein aantal unieke vlugtige verbindings was in die geïnfekteerde vatbare saailinge teenwoordig in vergelyking met die geïnfekteerde vatbare saailinge met groen blaar vlugtige verbindings wat in beide behandelings teenwoordig was. Dit was voorheen bewys dat sommige van die geïdentifiseerde vlugtige verbindings betrokke was in die verdedigingsrespons van plante.