Above ground plant interactions:
Consequences for growth and volatile emission

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Above-ground plant interactions:
consequences for growth and volatile emission

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General introduction

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Plant competition

The environment that plants grow in can be highly dynamic through a plant's lifetime. In many cases, plants have to compete with other plants in their direct surroundings for a limited pool of resources. Competitive interactions among plants shape vegetation composition and control biodiversity (Goldberg & Barton, 1992; Hautier et al., 2009), making it one of the most important processes for the development of local vegetation patterns.

To compete successfully, plants exploit a range of phenotypic responses that enhance resource capture and thus increase their fitness during competition (Callaway et al., 2003; Violle et al., 2009). In order to deal with proximate neighbours, plants have developed different strategies (reviewed in Novoplansky, 2009; Pierik et al., 2011). While competing, plants can i) escape the effects of neighbours by outgrowing them, e.g. through shade avoidance responses to escape shading by nearby plants (reviewed in Franklin, 2008), ii) suppress neighbours, e.g. by emitting compounds that inhibit their development (reviewed by Inderjit et al., 2011)) or iii) tolerate the impact of neighbours on available resource levels, e.g. shade tolerance of forest understory species (reviewed in Valladares & Niinemets, 2008). For plants to respond adequately through the above-mentioned strategies, in all cases rapid detection of neighbours is essential (reviewed in Pierik et al., 2011).

Depending on which resources plants are competing for, more carbon will be allocated to roots (competition for nutrients or water) or shoots (competition for light). When plants for example compete for nutrients, it is thought that root proliferation into nutrient-rich patches and a high total root length contribute to nutrient capture (reviewed by Hodge, 2004; Kembel & Cahill, 2005; Mommer et al., 2011), thereby determining part of the competitive vigour. During competition for light, shade intolerant species invest carbon and energy in so-called 'shade avoidance' responses, which include upward movement of leaves, enhanced elongation of internodes and petioles, and increased apical dominance, which

all function to position the photosynthesizing leaves in the well-lit, upper parts of the vegetation and away from the shade cast by neighbouring plants (reviewed in Franklin, 2008; Vandenbussche et al., 2005).

Shade avoidance: growing towards the light

The above-mentioned competitive behaviour is activated upon sensing of neighbour detection cues, such as a reduction in the photosynthetically active radiation (PAR), blue light or the ratio between red (R) and far-red (FR) light. Plants absorb mainly red and blue light for photosynthesis, while particularly far-red light, but to a lesser extent also green light, are transmitted through or reflected by photosynthesizing leaves. Whereas changes in blue light availability are perceived by the photoreceptor families of cryptochromes and phototropins, changes in R:FR balances are perceived by the plant's phytochrome photoreceptors.

Low R:FR perception and signal transduction

Resulting from R absorption and FR reflection and transmittance by leaves, the ratio between red and far-red light (R:FR) is reduced in a dense vegetation. A reduced R:FR ratio in the horizontally reflected light has been described as the earliest cue to detect nearby neighbours, even before actual shading takes place. Indeed, plants respond strongly to reduced R:FR ratios in a background with normal light intensity (Morgan & Smith, 1978). Classic responses of vegetative plants to low R:FR ratios include the shade avoidance syndrome (SAS) which encompasses such traits as enhanced hypocotyl growth, internode and petiole elongation, upward leaf movement (hyponasty) and increased apical dominance.
Changes in the R:FR ratio are perceived by the phytochrome photoreceptors, which in the model species Arabidopsis (*Arabidopsis thaliana*), constitute a family of five members; phytochrome A-E (PhyA-E). Of these, Phy, D & E are involved in regulating shade avoidance responses. Phytochromes have two forms: a biologically active (Pfr) and inactive (Pr) form. The active form has an absorption peak at a wavelength of 730 nm (which is far-red light) and absorption of light at this wavelength photoconverts Pfr into the inactive Pr. Pr has an absorption peak at a wavelength of 665 nm and converts upon absorption into Pfr (reviewed in Smith, 2000). Thus, a reduction in R:FR will result in a relatively large pool of inactivated phytochrome. Inactive Pr is located in the plant cell's cytosol, while activated Pfr is transported to the nucleus of a cell, where it interacts with phytochrome interacting factors (PIFs). These PIFs and PIF-like (PIL) proteins are members of the basic-helix-loop-helix (bHLH) family of transcription factors and they bind to DNA to regulate transcription of genes

associated with the induction of shade avoidance responses (Duek & Fankhauser, 2005; Huq et al., 2002; Leivar et al., 2008; Lorrain et al., 2008). Responses can be very rapid as is illustrated by the observation that expression of the shade avoidance marker gene *PIL1*, is enhanced within minutes after exposure to R:FR conditions (Salter et al., 2003). In addition to upregulation of *PIL1*, a reduction in R:FR leads to rapid induction of the expression of many other genes (Sessa et al., 2005) and a fast response to the changing light conditions.

One of the first identified up-regulated genes upon low R:FR exposure is *ATHB2*, a homeobox transcription factor (Carabelli et al., 1996; Carabelli et al., 1993). Expression of HFR1, a gene encoding a non-DNA binding bHLH protein, is up-regulated 15-fold after 1h low R:FR exposure (Sessa et al., 2005). Interestingly, *hfr1* knockout mutants are growing faster than wild type plants, implying that HFR1 is a negative regulator of plant growth. Upward leaf movement and stem and petiole elongation are primarily turgor-driven processes of cell expansion that rely on increasing extensibility of cell walls (Cosgrove, 2000). Expression of genes encoding endotransglucosylase/hydrolases (XTHs), a gene family that is known to be involved in cell wall loosening (Cosgrove et al., 2002) is up-regulated during shaded conditions (Sasidharan et al., 2010).

Perception of blue light and low photosynthetic active radiation (PAR)

Beside the absorption of red light, plants also typically absorb blue light. In dense stands, stems perceive reductions in blue light before actual shading of leaves. *Datura ferox* responds to reductions in blue light with enhanced internode elongation (Ballaré et al., 1991). In tobacco, reduced blue light availability leads to increased leaf hyponasty and internode elongation (Pierik et al., 2004a). Arabidopsis has two cryptochromes (Cry1 and Cry2) that are phosphorylated upon blue light perception (reviewed by Lin & Shalitin, 2003). Cry1 is mainly active at higher fluency rates, while Cry2 displays greater stability at lower fluency rates (Lin et al., 1998). It is suggested that both cryptochromes inhibit elongation growth upon blue light exposure with a partially redundant role for Cry2 (Mazzella et al., 2001). However, Cry2 is also known to interact with phytochromes (Mas et al., 2000). Phototropins are another family of blue light receptors that consists of two members: Phot1 and Phot2. They are involved in phototropic bending of shoots and in responses that optimize the photosynthethic efficiency of plants, such as stomatal opening and chloroplast movements (Christie, 2007). Phototropism can be helpful for plants in a canopy to growth towards patches with higher light availability.

At lower strata in dense vegetation, the total light availability and photosynthetically active radiation (PAR) will decrease. Plants growing in the understory of dense vegetation are exposed to reduced light intensity. Plant responses to a reduced light intensity without qualitative changes are somewhat ambiguous. In *Datura ferox*, reduced PAR does not lead to enhanced internodal lengths (Ballaré et al., 1991). However, reduced PAR does induce a hyponastic response in Arabidopsis (Millenaar et al., 2009) and can enhance elongation growth in stems of tobacco (Pierik et al., 2004a).

Ethylene

Although the earlier mentioned light signals are now well-established signals to detect nearby vegetation, it has been suggested that the volatile hormone ethylene can also serve as a neighbour detection cue (Pierik et al., 2004b). Ethylene is released by nearly all plant species (reviewed in Pierik et al., 2006; Jackson, 2008) and was found to accumulate in the boundary layer in dense stands of greenhouse-grown tobacco plants (*Nicotiana tabacum*) (Pierik et al., 2004b).

Ethylene is a well-studied volatile plant hormone that has long been known to control fruit ripening (Barry & Giovannonni, 2007) and flower senescence (Jones, 2008; Jones et al., 2005). It also has various roles in disease resistance (Dahl & Baldwin, 2007; van Loon et al., 2006; Pieterse et al., 2012) and growth regulation (Pierik et al., 2006, 2007; Bailey-Serres & Voesenek, 2008). Studies on tobacco showed that ethylene could be key to the appropriate timing of shade avoidance responses to close neighbours. Standardly grown individual tobacco plants were exposed to ethylene levels, similar to those that were recorded in the still canopy air, and these levels were found to induce shade avoidance responses, such as upward leaf movement and stem elongation (Pierik et al., 2004b). Furthermore, transgenic plants that expressed the mutant *etr1-1* receptor allele from the Arabidopsis *etr1-1* mutant that rendered them insensitive to ethylene, displayed significantly delayed shade avoidance responses to neighbours (Pierik et al., 2003). These transgenic plants were outcompeted by wild-type neighbours that did display appropriately timed shade avoidance responses. The competitive ability of these ethylene-insensitive plants could be restored by an end-of-day FR light pre-treatment that induced a mild shade avoidance phenotype before the onset of competition, thus showing that the ethylene insensitive plants performed poorly because of their poor shade avoidance reactions, rather than because of putative uncharacterized pleiotropic effects (Pierik et al., 2003). In Arabidopsis low R:FR conditions stimulate ethylene emissions (Pierik et

al., 2009) and ethylene plays a role low R:FR-induced petiole elongation (Pierik et al., 2009). Interestingly, blue light depletion can also stimulate shoot elongation, but in Arabidopsis, this does not coincide with increased ethylene emissions (Pierik et al., 2009). In tobacco, however, low blue light-induced internode elongation is entirely absent in ethylene-insensitive plants, further underlining the importance of light-ethylene crosstalk. It has also been recently shown that exogenous ethylene, produced by neighbouring plants, can reduce root growth of a target plant in a petri dish design (Inderjit et al., 2009). Ethylene is produced in roots and inhibits root elongation in most of the plant species studied so far (Pierik et al., 2007; Visser & Pierik, 2007), but it is unknown whether ethylene emitted by the roots of one plant can affect root growth of neighbouring plants in the soil. Elevated ethylene emission appears to be a general response to low R:FR for several species from different families, suggesting that ethylene involvement in plant competition is a general phenomenon (e.g. Finlayson et al., 1999; Foo et al., 2006; Kurepin et al., 2006a; Pierik et al., 2006). Moreover, ethylene plays a role in the local microtubule reorientation and cell expansion that are involved in hyponastic leaf growth (Polko et al., in press).

Competition impacts on VOC emissions

Over the last decade reports started to emerge showing that the emissions of various VOCs other than ethylene, such as mono- and sesquiterpenes, are affected by competition, as well as by abiotic stress factors that typically occur during competition, such as deficiencies in water, nutrient or light availibility (e.g. Gouinguene & Turlings, 2002).
Nutrients become depleted in soils that support high plant densities. Maize plants (*Zea mays*) that are grown on nutrient-depleted soil produce lower amounts of total VOCs upon herbivore induction than do maize plants growing on fertilized soil (Gouinguene & Turlings, 2002).

However, although the total sum of VOCs in this example is reduced owing to nutrient deficiency, particular compounds, such as the monoterpenoid linalool can be increased. Another study in maize plants showed enhanced emissions of volicitin (a VOC elicitor)-induced sesquiterpenes when grown on soil with reduced nitrogen content (Schmelz et al., 2003). A study of leaf terpene concentrations found that the leaf terpene concentration of Aleppo pine (*Pinus halepensis*) increased with an increasing amount of nitrogen or phosphorus in the soil, whereas soil nitrogen and phosphorus content did not affect leaf terpene concentration of rock rose (*Cistus albidus*) and rosemary (*Rosmarinus officinalis*) (Ormeño et al., 2008). Although the extent to which leaf terpene concentrations affect

volatile terpene emission in these two studies was not investigated, the results indicate a strong variability of terpene production between species under varying soil nutrient contents.

In addition to nutrients, light intensity is strongly reduced in dense canopies and VOC emissions are tightly correlated with light availability. For example, VOC emission of lima bean (*Phaseolus lunatus*), induced by mechanical damage, occurs mainly during the light period, even when plants were damaged during the dark period (Arimura et al., 2008), indicating a light dependency of VOC release. In maize, total VOC emissions of healthy, undamaged plants are only mildly affected by light intensity, whereas VOC emissions of herbivore-attacked plants are strongly light dependent (Gouinguene & Turlings, 2002). Perhaps more importantly, different VOCs show different emission patterns in response to light, leading to a correlation between the composition of the VOC blend and a change in light intensity (Gouinguene & Turlings, 2002). The light dependency of VOC emissions might be related to a variety of factors, such as stomatal conductance, evaporation rates of the compounds (Niinemets & Reichstein, 2003a, 2003b; Niinemets et al., 2004) and rates of photosynthesis (Grote, 2007; Niinemets et al., 2002). Contrary to control through light intensity, little is known about VOC emission under different qualities of light, except for the volatile hormone ethylene. In addition to light and nutrients, plants also compete for water. Terpene emissions were up-regulated for several Mediterranean species that grow in monocultures under natural environmental conditions (Ormeño et al., 2007a). On calcareous soil, *P. halepensis*, *C. albidus* and *Q. coccifera* showed upregulation of mono- and sesquiterpene emission under intraspecific competition.

However, emission patterns for specific volatiles differed between species. For example, α-pinene emission was up-regulated in *Q. coccifera* and *P. halepensis*, but not in *C. albidus*. When these species were not competing with conspecific neighbours but with plants from other species, terpene emissions were generally reduced (Ormeño et al., 2007b). These observations indicate that terpene emissions are affected by competition, but the details of this regulation depend on the plant species and identity of the compound.

Anticipating neighbours: VOCs can affect plant growth and competition

As indicated in the previous section, VOC emissions differ for competing plants and non-competing individuals, in both quantitative and qualitative aspects. Because plants can perceive variations in VOC emissions, at least VOCs induced upon herbivory (Baldwin et al., 2006), this opens up a novel form of interactions between competing plants.

In dense vegetation where plants grow closer together than in open stands, chances are higher for VOCs to affect neighbouring plants as they are more likely to reach physiologically relevant concentrations, as has been shown for ethylene in dense tobacco stands (Pierik et al., 2004b). VOCs can affect neighbouring plants in two ways: they can (i) cause allelopathic effects (e.g. inhibit growth or developmental programs); and (ii) be exploited by neighbouring plants as a cue for the presence of proximate competitors, thus inducing or priming responses that increase the competitive power of the 'eavesdropping' neighbour (Dicke et al., 2003; Heil & Karban, 2010). There are several studies that show evidence for VOC-mediated control of neighbour plant growth. However, only a few of these studies have been performed under natural field conditions.
Therefore, for studies that were performed under laboratory conditions, it remains unknown whether the reported effects of VOCs would occur in neighbouring plants under natural field conditions, for example because concentrations will not always be high enough for an effect to occur (Barney et al., 2009).

VOCs as allelochemicals

Allelopathic effects of root-emitted VOCs, such as the monoterpenoid α-pinene, have been shown in several plant species. For example, holm oak (Asensio et al., 2007) and purple sage (*Salvia leucophylla*) (Nishida et al., 2005) emit α-pinene belowground. In a study of five different species, α-pinene application appeared to inhibit seed germination of three species. Early root growth was inhibited in all five species studied and was accompanied by increased levels of oxidative stress in the roots (Singh et al., 2006). In addition, a range of other *S. leucophylla* root emitted monoterpenoid VOCs, such as camphor, camphene, 1,8-cineole and β-pinene, inhibited germination and growth of rapeseed (*Brassica campestris*), when applied externally. Those effects were restricted to the root apical meristem and did not affect the mitotic index in the shoot apical region, indicating tissue specificity for the allelopathic effects of these volatile compounds (Nishida et al., 2005). The authors suggest that these allelopathic effects of VOCs emitted by this *Salvia* species prevent other species from entering Salvia vegetation, thus avoiding interspecific competition (Nishida et al., 2005). The seed germination of potential future competitors can also be inhibited by shoot-emitted VOCs. For example, volatile compounds that are emitted by snapdragon (*Antirrhinum majus*) flowers inhibit root growth in neighbouring Arabidopsis seedlings under laboratory conditions (Horiuchi et al., 2007). This might be caused by methyl benzoate, a component of the floral VOC blend of *A. majus*, which appears to affect the expression of genes associated with various classes

of growth-regulating hormones, such as auxin and cytokinin (Horiuchi et al., 2007). A study of sagebrush (*Artemisia tridentata*) showed that, upon clipping of foliage, the germination of neighbouring seeds of different species is inhibited. Conditions under which air contact was avoided, but transmittance through the soil was permitted, prevented these inhibitory effects on germination (Karban, 2007). These data indicate that, upon foliage clipping, sagebrush emits VOCs that inhibit germination of neighbouring seeds, thus preventing future competition. Formally, it cannot be excluded that the reduced germination is, rather than an allelopathic effect of the VOC emitter, an adaptive strategy of the receiving seeds, which by delaying germination upon detection of herbivore-induced VOCs might prevent direct challenges with herbivores in the early seedling stage.

Eavesdropping on VOCs for neighbour detection

As described for ethylene earlier in this chapter, VOCs can also act as a neighbour detection signal and induce growth responses that are adaptive (i.e. confer a fitness advantage) in neighbouring plants. Similar to the classic neighbour detection signals, such as reduced R:FR ratio, ethylene emissions hold little information about the competitive vigour of neighbouring competitors since most plants produce and emit this volatile.

Individual volatile compounds (such as ethylene) or volatile blends might hold potential for more sophisticated neighbour detection and even discrimination between different neighbour identities, because different plant species typically produce different blends of VOCs. An example comes from a greenhouse study on two barley (*Hordeum vulgare*) cultivars, Alva and Kara (Ninkovic, 2003). The Kara cultivar allocates more biomass to its roots when it is exposed to volatiles from the barley cultivar Alva, compared with plants exposed to clean air or to Kara volatiles. Total biomass did not differ between the three treatments, but Kara plants exposed to Alva volatiles had a higher specific leaf area (m2 kg-1 leaf), a response that also occurs upon exposure to low light conditions, such as occurs in shaded leaves in a canopy. Thus, exposure to VOCs from the same cultivar had no effect, whereas exposure to the VOC blend of a different cultivar did result in phenotypic adjustments. This suggests that there are differences between the two volatile blends that can be detected by plants, although no evidence is available on VOC perception systems that would be required for this sophisticated discrimination between VOC blends.

Another example of VOC-mediated discrimination of neighbour identity comes from a study of host localization by golden dodder (*Cuscuta pentagona*), a parasitic plant (Runyon et al., 2006). It was shown that

seedlings of this species find their host through volatile cues emitted by the host plant, rather than through other signals, such as light. Even more strikingly, seedlings could discriminate between different neighbouring species, again based on the relative VOC blends from the different species, thus ensuring growth towards a host (tomato; *Solanum Lycopersicon*) rather than a non-host (wheat; *Triticum aestivum*, in these experiments). Discrimination between the host and non-host VOC blends might be based on a combination of positive responses to some volatiles from tomato [e.g. β-phellandrene, β-myrcene (also produced by wheat) and α-pinene] and repellent effects of the volatile (*Z*)-3-hexenyl acetate (Runyon et al., 2006). Taken together, a multitude of different signals exists that can hold information about neighbours. Some, such as low R:FR ratio, are reliable indicators of proximate neighbours but hold little information about, for example, growth rate. Other signals, such as the variety of VOC blends, might contain detailed information about neighbour identity, but might be less reliable indicators of competition because they are sensitive to disturbing factors, such as wind. Exploiting the combination of different signals from neighbours might be the most reliable way for plants to evaluate the threat of competition.

Conflict of interest: interaction between competition and defence

Various recent reports have shown that induced emission of VOCs upon wounding or attack by herbivores can function as within-plant signals to induce defence responses in remote plant parts, such as branches, that are not yet attacked (e.g. Frost et al., 2007, 2008; Heil & Silva Bueno, 2007). When plants are growing in dense stands, the likelihood of these volatiles reaching biologically meaningful concentrations in the air between nearby neighbours is relatively high because inter-plant distances are small and wind velocities are low. Therefore, under such dense conditions, herbivore-induced VOCs might be relatively likely to be sensed by eavesdropping neighbours, a phenomenon that can occur among conspecifics as well as among individuals from different species (Karban, 2001; Karban et al., 2004; Baldwin et al., 2006; Dicke & Bruin, 2001) . The potential for VOC-induced defence against herbivores is relatively high in dense stands with strong competitive interactions between individuals. A second implication might be that herbivory-induced VOC accumulations could conflict with competitive strategies that involve growth investments, because some of these volatiles, such as methyl-jasmonate (Me-JA) and methyl salicylate, can inhibit growth or induce the production of allelopathic compounds (Bi et al., 2007; Hummel et al., 2009; Staswick et al., 1992).
The interplay between competition and defence against herbivores

has been studied recently in wild tobacco (*Nicotiana longiflora*) and Arabidopsis. Activation of an adequate defence response depends on light intensity (Genoud et al., 2002) and phytochrome-induced shade avoidance responses appeared to dominate over responses that enhance defence against herbivores (Izaguirre et al., 2006; Moreno et al., 2009) and pathogens (Cerrudo et al., 2012; de Wit, 2012).
Importantly, these studies did not account for potential effects of low R:FR ratio on volatile emission and/or consequences for VOC signalling between individuals. In contrary to other wavelengths, plants grown under red light were more resistant to *Spaerotheca fuliginea* (Wang et al., 2010). Accordingly, reductions in the R:FR conditions lead to reduced defence responses. Constitutive shade avoiding *phyA-phyB* double mutants show less induction of the PATHOGENESIS RELATED 1 (PR1) gene than wild type plants upon infection with the bacterium *Pseudomonas syringae*, and bacterial proliferation was enhanced in the mutant (Genoud et al., 2002). Plants grown at low R:FR conditions did accumulate lower amounts of phenolic compounds upon jasmonic acid (JA) treatment and low R:FR exposure reduced the induction of JA dependent defence genes (Izaguirre et al, 2006). In low R:FR conditions, plants seem to be desensitized for JA (Moreno et al., 2009). This desensitization does not seem to involve the well-established route of salicylic acid-mediated inhibition of the JA-induced neighbour pathway through NPR1 (Non-expressor of PR genes), but involves regulation of the JAZ10 (JAsmonate Zim-domain protein 10) transcriptional suppressor (Cerrudo et al., 2012). Larval growth of the generalist herbivore *Spodoptera frugiperda* increases more on plants exposed to low R:FR conditions and on *phyB* mutants (Moreno et al., 2009) compared to control and wild type plants, suggesting that suppression of plant defence under shading conditions is regulated through phytochrome signalling. Application of JA and MeJA is not only effective in inducing direct neighbours in plant leaves, but can also mimic the emission of herbivore-induced VOCs (e.g. Snoeren et al., 2009; Herde et al., 2008; Bruce et al., 2008; Faldt et al., 2003; Chen et al., 2003).

Considering the suppression of plant defence when in shaded conditions through desensitization to JA and the role of (Me)JA in the production and emission of VOCs, it is possible that (Me)JA-induced VOC emissions (Snoeren et al., 2009) are reduced under low R:FR conditions. If the trade-off between competition and defence (reviewed in Ballaré et al., 2012) also occurs at the level of VOCs, growth-inhibiting effects of herbivore-induced volatiles might be less likely to occur. During simultaneous exposure to competition and herbivory, the multitude of different signal combinations indicating herbivory and competition provides a more subtle, complete and reliable picture of the competitive environment of the plant.

Outline of this thesis

In this thesis studies of plant neighbour detection will be presented in relation to light signals, ethylene and other VOCs. Furthermore, interactions between light quality and VOC emission, including consequences for plant-plant and plant-insect interactions, have been investigated.
In chapter 2, experiments on the timing of competition cues and responses in dense Arabidopsis vegetations are presented. Hyponasty was found to be the earliest visible neighbour response and occurred at an earlier time point than low R:FR-enhanced petiole elongation and shade avoidance marker gene regulation. A virtual canopy model showed that hyponasty is required in Arabidopsis stands to create reduced R:FR ratio's in reflected light. Finally, we demonstrated that early hyponasty is not induced by light signals, but by touching leaf tips; a novel neighbour detection system.
In chapter 3, interaction between the volatile plant hormone ethylene and a reduction in R:FR was further investigated. Here, it is described that perception of ethylene is required for low R:FR induced petiole elongation, when the R:FR ratio is mildly reduced. At a more severely reduced R:FR ratio, perception of ethylene enhances the rate of hyponastic leaf movement.
Chapter 4 describes effects of neighbour-induced alterations in the light environment on VOC emissions in Arabidopsis. Light signals that represent different degrees of competition for light led to similar reductions in the emission of VOCs. The emission of methyl-jasmonate-induced VOCs, was found to be reduced in low R:FR conditions and this affected plant preference of the specialist herbivore *Pieris brassicae*.
In chapter 5, the effect of low R:FR conditions on volatile effects between plants is demonstrated in barley (*Hordeum vulgare*). When volatile-emitting plants were exposed to low R:FR, VOC emissions were reduced. As a consequence, effects on carbon allocation by VOCs from emitter plants of one cultivar (Alva) on receivers plant from another (Kara) depend highly on the R:FR conditions of the emitter. These data indicate the importance of the light environment on chemical interactions between plants.

A summarizing discussion on the work described in this thesis is given in chapter 6.

CHAPTER

1 2 3 4 5 6

Plant neighbour detection through touching leaf tips precedes phytochrome-mediated neighbour detection

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Abstract

Plants in dense vegetation compete for resources, including light, and optimize their growth based on neighbour detection cues. The best studied of such behaviors is the shade-avoidance syndrome that positions leaves in optimally lit zones of a vegetation. Although proximate vegetation is known to be sensed through a reduced ratio between red and far-red light, we show here through computational modeling and manipulative experiments that leaves of the rosette species *Arabidopsis thaliana* first need to move upward to generate sufficient light reflection potential for subsequent occurrence and perception of a reduced red to far-red ratio. This early hyponastic leaf growth response is not induced by known neighbour detection cues under both climate chamber and natural sunlight conditions, and we identify a novel way for plants to detect future competitors through touching of leaf tips. This signal occurs before the detection of light signals and appears to be the earliest means of aboveground plant–plant signalling in horizontally growing rosette plants.

Introduction

Plant growth in dense vegetations is dominated by a fierce battle over resources. Whereas plants compete belowground for water and nutrient availability, plants compete aboveground for light. In competition for light, small size inequalities can have major effects on light capture: when a plant is overgrown by a proximate neighbour, access to light is limited and competitive power strongly reduced (Ballaré et al., 1988; Pierik et al., 2003;

Schmitt et al., 1995). Plants can detect changes in the light environment with the phytochrome family of photoreceptors. These photoreceptors respond to a reduction in the ratio between red (R) and far-red (FR) light, which is typically caused by neighbouring plants due to absorption of red light and reflection of far-red light. A change in the R:FR is described as the earliest plant neighbour detection signal, since R:FR is decreased even before true shading occurs through overlap of leaves (Ballaré et al., 1990). Low R:FR induces shade-avoidance responses such as upward leaf movement (hyponasty) and stem elongation that secure light capture during subsequent competition with neighbouring plants (Franklin, 2008; Vandenbussche et al., 2005). Accordingly, when plants grow under low R:FR in a high PAR environment, this already induces shade-avoidance responses (Morgan et al., 1980; Morgan & Smith, 1978). When the R:FR ratio is decreased, phytochrome B (phyB) is photo-converted from its active to inactive form. This conversion inhibits phyB from binding to phytochrome interacting factors (PIFs), which are typically degraded upon phy-binding. As a consequence of phytochrome inactivation in low R:FR conditions, PIFs are not degraded and can transcriptionally activate the signalling cascade that leads to shade avoidance (Li et al., 2012; Lorrain et al., 2008). Shade avoidance responses such as upward leaf movement and stem and petiole elongation are primarily turgor-driven processes of cell expansion that rely on increasing extensibility of cell walls (Cosgrove, 2000). The family of endotransglucosylase/hydrolases (XTHs) is known to be involved in cell wall loosening (Cosgrove et al., 2005) and the expression of genes coding for several XTHs is up-regulated in shading conditions (Sasidharan et al., 2010).

Although some additional above-ground neighbour detection signals are known, e.g., blue light depletion (Ballaré et al., 1991; Pierik et al., 2004a), accumulation of the volatile plant hormone ethylene (Pierik et al., 2004) and even mechanical stimuli (Nagashima & Hikosaka, 2012), none of these acts as early as a decrease in the reflected R:FR. The paradigm of decreased R:FR as the earliest neighbour-detection signal in competition for light has been a breakthrough in mechanistic plant competition research. It is important to realize, however, that this paradigm is based on research on stem-forming forbs and trees (Ballaré et al., 1987; Gilbert et al., 2001; Kasperbauer, 1971), which are species that show pronounced vertical height growth. As a result these species form a vertical structure that has the capacity to reflect FR light horizontally. However, R:FR reflection has not been studied in dense stands of plants that lack an appreciable height growth before competition in the vegetative life stage, e.g. rosette species such as *Arabidopsis thaliana* and various other species. Here we study early neighbour detection in dense stands of Arabidopsis. Hypo-

nasty appears to be the earliest shade-avoidance response and occurs exclusively in leaves that touch neighbouring leaf tips, before a physiologically meaningful decrease in R:FR. Using a combination of manipulative experiments and computational modeling, we introduce physical touch as a signal in neighbour detection that is required to establish sufficient FR reflection to generate a low R:FR light cue in rosette canopies under both artificial and natural light quality conditions.

Materials and Methods

Plant growth and measurements

Arabidopsis thaliana wild-type Columbia-0 (Col-0), *wei8-1*, *pif4pif5*, *pif7-1*, *rot3-1*, *pin3-3*, *etr1-4*, *aos*, *jar1-1*, *coi1-1*, *npr1-1*, and *bri1- 1* (all mutants in Col-0 background) (Bleecker et al., 1988; Cao et al., 1997; Clouse et al., 1996; Friml et al., 2002; Kim et al., 1998; Leivar et al., 2008; Lorrain et al., 2008; Park et al., 2002; Staswick et al., 1998; Stepanova et al., 2008; Xie et al., 1998) were stratified (dark, 4 °C, 3 d) and subsequently grown in soil with additional nutrients (Millenaar et al., 2005) in a growth room [9-h light period (200 µmol m–2·s–1 PAR (photosynthetically active radiation), R:FR 2.1 or 1.2; Philips HPI-T Plus, 400 W), 20 °C ± 2 °C, 70% RH ± 5%]. After 10 d, seedlings were transferred to individual 70-mL pots or to tree trays with individual pots of 19 mL (2.2 × 2.2 × 6.2 cm) to create dense stands (2,066 plants m-2) of 7 × 7 plants (2.2 cm between the nearest neighbouring plants). For canopy development time-series, two different canopies were harvested and analyzed at each time point. Only the inner nine plants were measured; the outer rows served to minimize edge effects. To synchronize graphs between genotypes and treatments, plant development in canopies was plotted against leaf LAI (cm2 leaf area cm-2 soil area available per plant), which increased with time, a standard way to represent competition intensity (Ballaré et al., 1987, 1991). Single plants were plotted against the LAI of canopies and represent control of precisely the same age as the corresponding plants in a canopy. Plants of 24 d old were used for all experiments with individually grown plants, which is the age at which canopy-grown plants show a strong increase in leaf angle. For touch experiments, three transparent tags (polycyclical olefin) were placed in the soil such that three leaf tips were just touching the tag. The third-youngest leaf was measured for hyponasty and elongation from pictures using ImageJ (Abràmoff et al., 2004) (measurements on individual plants), a digital caliper and a protractor (for canopy plants). Leaf areas were measured with a Li-3100 Area Meter (LI-COR) to calculate LAI. Lamina shape was assessed for modeling by measuring blade width as a function of distance to the blade tip, in intervals of 5 mm along

the midrib of the blade, on five randomly chosen leaves. Hyponasty kinetics were determined in continuous light from time-lapse images as in Millenaar et al. (2005) using a computer-controlled moving digital camera that takes pictures at regular intervals from the side, and ImageJ to record angles of target petioles from these images.

Light manipulations and recordings

R:FR ratio manipulations in growth chamber compartments occurred through supplemental FR LEDs (λ = 730 nm; Philips Green Power) in a control white-light background of 110 µmol m–2·s–1 PAR. Low blue light was created with a blue light-absorbing filter (LEE 010 Medium Yellow), leaving 90 µmol m–2·s–1 PAR after filtering. Control plants were exposed to the same light intensity. Light quality inside canopies was manipulated with narrow strips of red light-emitting LEDs (λ = 620–635 nm) to increase R:FR, or green (λ = 520–530 nm) LEDs, as a control, that were placed in between the rows of plants. Measurements of reflected light inside canopies were taken with a LI-COR 1800 spectroradiometer using a glass fiber with cosine corrector. Representative scans of the light spectrum in control light and in a hyponastic canopy of LAI 2.54 are shown in figure S2.1. The R:FR was calculated from the irradiance within the 654- to 664-nm waveband for R and 724–734 nm for FR. Blue light was calculated as the irradiance within a 400- to 500-nm waveband. Every reading was taken four times and the average calculated.

Simulations

A functional-structural plant model (Vos et al., 2010) was built to simulate *Arabidopsis thaliana* canopies (2,066 plants/m2) and the R:FR ratio within this canopy (using the simulation platform GroIMP and its radiation model) (Hemmerling et al., 2008), that used leaf appearance rate, blade extension rate, petiole extension rate, blade shape, blade size, petiole size, and phyllotactic angle as input (most parameter values were obtained from dedicated experiments, some were from the literature, i.e. Mundermann et al., 2005). In simulations used to calculate R:FR in a developing canopy, leaf hyponastic angle was taken as input and calibrated from our experimental data. In simulations used to assess the effect of canopy structure on canopy R:FR, leaf hyponastic angle of all leaves was increased from 0° to 80° in steps of 5°, in static canopies at LAI = 0.65. Canopy development was simulated by arranging 49 individual plants in a regular 7 × 7 grid similar to the experimental setup. The middle 3 × 3 plants were used for analysis to avoid edge effects. Simulations of single plants were performed nine times to reach the same sample size. Orientation in the x-y plane of simulated plants was chosen at random. The radiation model simulated light rays emitted from a virtual light source

above the simulated plant(s) and traced them through the canopy, taking into account differential scattering and absorption by the plant organs of red and far-red light as experimentally determined for Arabidopsis plants.

RNA isolation and RT-qPCR

Petioles of the third-youngest leaf were harvested separately and snap-frozen for storage at −80 °C. Petioles from at least three individual plants were pooled into one sample and homogenized, and total RNA was extracted using the Qiagen RNeasy Plant Mini Kit with on-column DNase treatment. cDNA was synthesized by 100 units of SuperScript III reverse transcriptase (Invitrogen) with random hexamers at 50 °C in a reaction volume of 20 µL. RT-qPCR was performed in a BioRad MyIQ single-color real-time PCR detection system using SYBR Green Supermix (BioRad). Gene-specific primers (Table S2.1) were designed using the Primer3Plus software (Untergasser et al., 2007) and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression (Livak & Schmittgen, 2001) with UBQ5 as internal standard.

Microarray analysis

Total RNA was extracted from homogenized material for three biological replicates of pooled petioles using the RNeasy Plant MiniKit with on-column DNA digestion (Qiagen) following the manufacturer's instructions. cDNA synthesis, cRNA synthesis, and hybridization to ATH1 Affymetrix Arabidopsis Gene Chips were performed by ServiceXS (authorized service provider, Affymetrix). The robust multi array analysis algorithm (RMA; Irizarry et al., 2003) was used to normalize expression values, and the empirical Bayes method (Smyth, 2004) and Benjamini and Hochberg multiple-testing correction (Benjamini & Hochberg, 1995) were used to assess differential expression in the Bioconductor packages in R (www.bioconductor.org). Genes with a threshold B value (empirical Bayes log odds of differential expression) of 2 were considered differentially expressed. Gene ontology (GO) analysis was done with the BiNGO plug-in of Cytoscape (Maere et al., 2005). Data are available at the NCBI gene expression and hybridization array data repository, Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo; accession no. GSE39010).

Statistics

Data were analyzed through one-way ANOVA with Bonferroni post hoc test, two-way ANOVA with Bonferroni post hoc test or Student's t test in IBM SPSS statistics 20.

Results

Early neighbour-induced hyponasty precedes a functional decrease in R:FR.

To study early neighbour detection in a rosette species, we studied above ground competition with neighbouring plants in the model plant *Arabidopsis thaliana*. Plants were grown at a density of 2,066 plants m-2 in individual pots to exclude belowground interactions. Over time (expressed through leaf area index (LAI): leaf area/soil area), high density-grown plants developed a typical shade-avoidance phenotype (fig. 2.1 A). A significant interaction effect was found between the effect of density and LAI ($p<0.001$). At an LAI of 0.65, plants growing in the canopy setup started to show increased petiole angles (hyponasty) compared with single-grown control plants (fig. 2.1 B), whereas enhanced petiole elongation of canopy-grown plants only occurred from an LAI of 1.27 onward (fig. 2.1 B). The R:FR in the light reflected from leaves within the canopy decreased most rapidly between LAI 0.5 and 1.0, and only decreased below 1.0 at LAI >2, when leaves were fully overlapping (fig. 2.1 B). At the LAI (0.65) when hyponasty first occurred, the R:FR in the reflected light had only decreased to 1.7. Interestingly, microarray analysis on petioles from canopy plants at LAI 1.0 showed no induced expression of genes known to be involved in shade avoidance (table S2.2), and as a consequence no Gene Ontology (GO) enrichment for shade avoidance or light signalling were found (table 2.1). Accordingly, a quantitative RT-PCR time course throughout canopy development indicated that expression of the low R:FR-inducible marker genes *ATHB2* and *XTH15* (Carabelli et al., 2007; Sasidharan et al., 2010) was not induced at the onset of hyponasty (LAI 0.65). These genes were only up-regulated after petiole elongation occurred in canopy plants at an LAI >1.27, which corresponded with an R:FR <1.3 (fig. 2.1 B and C). In conclusion, hyponastic growth is the earliest shade avoidance response in Arabidopsis canopies and occurs already after a relatively small decrease in R:FR (from 2.1 to 1.7) and before shade-avoidance marker genes are induced.

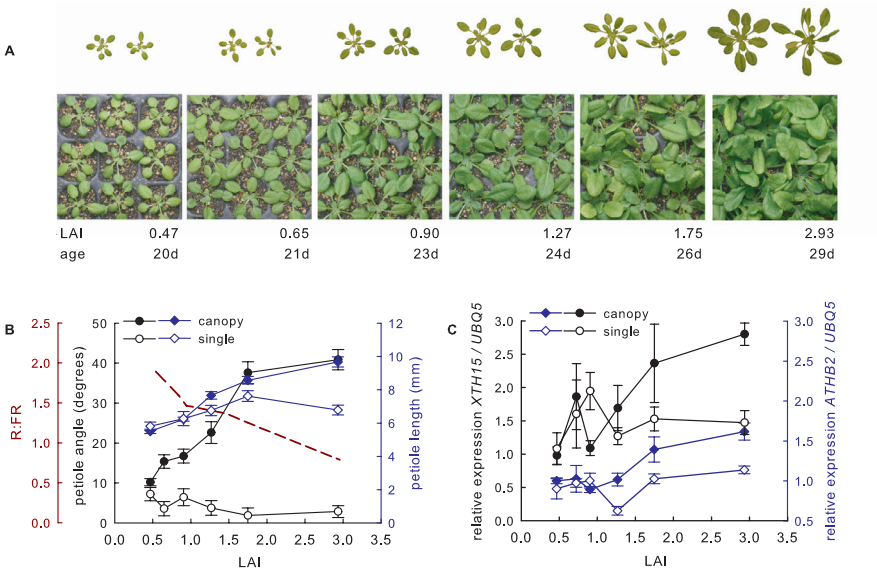


Figure 2.1 Shade-avoidance traits in developing Arabidopsis canopies. (A) Representative photographs of the inner nine plants of a canopy at different LAIs with above each canopy photo two individual rosettes of the same age, of which the left is always a single-grown, and the right is a canopy-grown plant. (B) R:FR of reflected light inside the canopy, petiole angles (left y axis, black lines) and lengths (right y axis, blue lines) of the third-youngest leaves of canopy-grown (filled symbols) and single-grown (open symbols) plants (n = 27 from three individual canopies for canopy plants, n = 9 for single-grown plants). Petiole angles from canopy plants are significantly different from single plants from LAI = 0.65 onward; petiole lengths from LAI = 1.27 onward (Student's t test, $P < 0.05$). (C) *XTH15* (left y axis, black lines) and *ATHB2* (right y axis, blue lines) relative expression (quantitative RT-PCR) in canopy-grown (filled symbols) and single grown (open symbols) plants (n = 4). Expression of both genes is significantly induced in canopy plants at LAI = 2.9 (Student's t test, $P < 0.05$). Data represent means ± SE. Data for single plants are plotted against canopy LAI and represent plants of the same age as their equivalent in the canopy at a specific LAI.

Table 2.1 Gene ontology analysis of differentially expressed genes in petioles of canopy-grown plants at LAI = 1.0

Gene ontology ID	Description	Corrected P value
	Response to stimulus	
9741	Response to brassinosteroid stimulus	1.77E-03
9617	Response to bacterium	1.30E-02
52543	Callose deposition in cell wall	2.15E-02
10200	Response to chitin	3.52E-02
9627	Systemic acquired resistance	3.70E-02
	Biological regulation	
7169	Transmembrane receptor protein tyrosine kinase signaling pathway	3.39E-02
169	Activation of MAPK activity involved in osmosensory signaling pathway	3.39E-02
60862	Negative regulation of floral organ abscission	3.39E-02
31349	Positive regulation of defense response	3.70E-02
	Metabolic process	
10120	Camalexin biosynthetic process	7.23E-03
6575	Cellular amino acid derivative metabolic process	4.04E-02
	Catalytic activity	
16491	Oxidoreductase activity	1.98E-02
20037	Heme binding	2.22E-02
4496	Mevalonate kinase activity	3.39E-02
	Cell part	
9505	Plant-type cell wall	5.58E-03
48046	Apoplast	2.15E-02
5886	Plasma membrane	3.83E-02

Neighbour-induced hyponasty is not induced by known neighbour detection signals.

Because the first measureable neighbour response occurred after a relatively small decrease in R:FR, we studied whether the decrease in R:FR from 2.1 (standard growth chamber light) to ~1.7 (LAI = 0.65) would be sufficient to induce the hyponastic response measured at LAI 0.65. We observed that even a slightly stronger reduction in R:FR (from 2.1 to 1.5) was insufficient to induce hyponasty in individually grown plants without neighbours, and that only when the R:FR ratio was further reduced to 1.2 or lower was hyponasty observed (fig. 2.2 A). Such strong R:FR reductions occurred only at LAI>1.5 when petiole elongation was also induced (fig. 2.1 B). This finding corresponds with the induction of the shade-avoidance marker genes (fig. 2.1 C) that were only up-regulated at LAIs at which the R:FR was below 1.3, and suggests that the R:FR in the canopy is not responsible for the early hyponastic response to neighbours. To verify that the early hyponastic response is not induced by a decrease in low R:FR, we used the wei8-1 mutant, which lacks functional TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) that acts in the auxin biosynthetic pathway required for shade-avoidance responses to low R:FR (Stepanova et al., 2008; Tao et al., 2008).

This mutant showed a wild-type early hyponastic response to neighbours in high density (fig. 2.2 B and C); a similar percentage of all leaves were hyponastic (petiole angle > 15°) in *wei8-1* and wild-type canopies, even though *wei8-1* does not show increased leaf angles in response to low R:FR (fig. 2.2 D). As final proof that early hyponasty was independent from a reduction in R:FR, we mounted R-emitting wide-angle light-emitting diodes (LED) lights between the dense plants (fig. 2.2 E), which at LAI = 0.6 restored the R:FR from 1.6 (control canopies) to 2.1 (background light). Elevated R:FR, however, did not reduce the number of hyponastic leaves at LAI 0.6, which was 15–20 % of all leaves in all canopies (fig. 2.2 F).

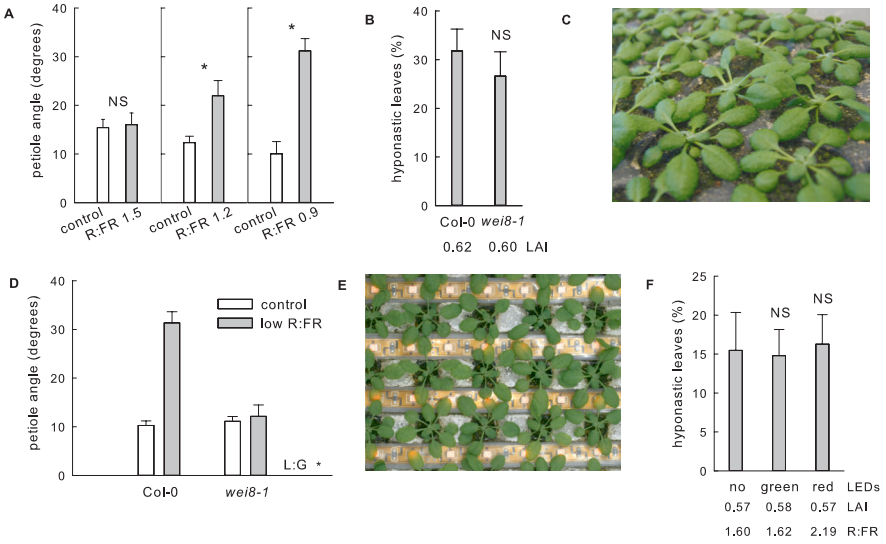


Figure 2.2 Mild reductions of R:FR are not responsible for early hyponasty in canopies. (A) Petiole angles after 6 h of exposure to R:FR = 1.5, R:FR = 1.2, and R:FR = 0.9 (n = 10). (B) Percentage from total leaves that are hyponastic in a Col-0 canopy and in a *wei8-1* canopy. (C) Representative photograph of hyponastic *wei8-1* canopy. (D) Petiole angles in the shade-avoidance mutant wei8-1 and its wild-type Col-0 after 6 h of control light or low R:FR (0.2). Asterisk indicates significant difference (two-way ANOVA, $P < 0.05$, n = 8). G, genotype; L, light treatment; L:G, interaction between light and genotype. (E) Representative photograph of a canopy with supplemental R LEDs. (F) Percentage from total leaves that are hyponastic in a canopy without LEDs, with green supplemental LEDs, and with red supplemental LEDs. Data represent means ± SE. Asterisk indicates significant difference (one-way ANOVA, $P < 0.05$, n = 18 plants from two individual canopies); NS, not significant.

Alternative early light signals to a reduced R:FR in a canopy environment would be reductions in the availability of blue light and total (PAR) light (fig. 2.3 A). Plants grown under comparable low light and low blue light conditions as observed in the canopy at LAI = 0.6 did however not show an increase in petiole angle (fig. 2.3 B & C), suggesting that these changes in light availability are not involved in early neighbour detection in this canopy system.

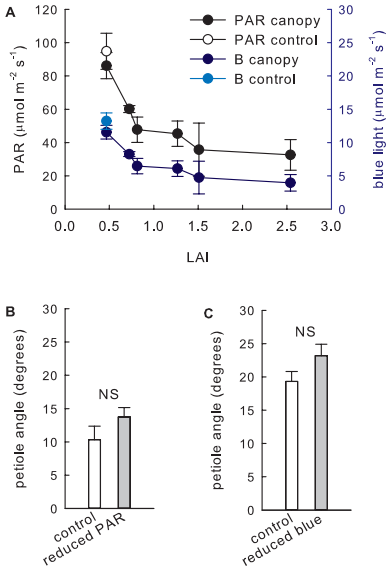


Figure 2.3 Perception of reduced (blue) light intensity does not play a role in the hyponastic response to neighbors. (A) Photosynthetically active radiation (PAR; left y axis, black lines) and blue light (right y axis, blue lines) intensity in the reflected light of canopies over time plotted against the leaf area index (LAI) of the canopies. White/light-blue symbol at the beginning of each plot represents the control value of the incoming light. Each data point represents the mean of four individual scans. (B) Petiole angles after 6 h of reduced PAR (30% reduction to 140 µmol m−2·s−1 vs. 180 µmol m−2·s−1 in control) and (C) reduced blue light (1 µmol m−2·s−1 vs. 17 µmol m−2·s−1 in control; in PAR of 90 µmol m−2·s−1 for both light treatments). NS, not significant (Student's t test, $P > 0.05$, $n = 10$).

Early hyponasty is induced by touching of neighbouring leaves.
We observed that the first leaves to become hyponastic were always those that were touching leaf tips from neighbouring plants. Instead of one leaf overgrowing the leaf of another plant, both leaves would move upward (fig. 2.4 A). Accordingly, all hyponastic leaves (petiole angle >15°) at LAI 0.6 were found to be touching a leaf from a neighbouring plant (fig. 2.4 D), suggesting that the early hyponastic response in canopies is induced through touching of leaves. Indeed, when two single-grown plants were placed next to each other with the leaf tips from the two different plants facing each other, a similar hyponastic response could be induced (fig. 2.4 B). To exclude the influence of a R:FR signal in this touch-induced hyponasty, we placed transparent tags at the end of leaf tips in the soil next to a single plant. The petioles grew upward when touching the tags and remained elevated after subsequent removal of the tags (fig. 2.4 E). The hyponastic response was only induced in touching leaves and not in systemic ones (fig. 2.4 A and C-E). It took approximately 4 h of continuous exposure to the touch stimulus (a transparent tag) for the leaves to start hyponastic growth (fig. 2.5). Repeated touching of leaf tips with a brush with 0.5 h or 1 h intervals did not result in elevated petioles, suggesting that the touch stimulus has to be continuous to induce hyponasty (fig. S2.2).

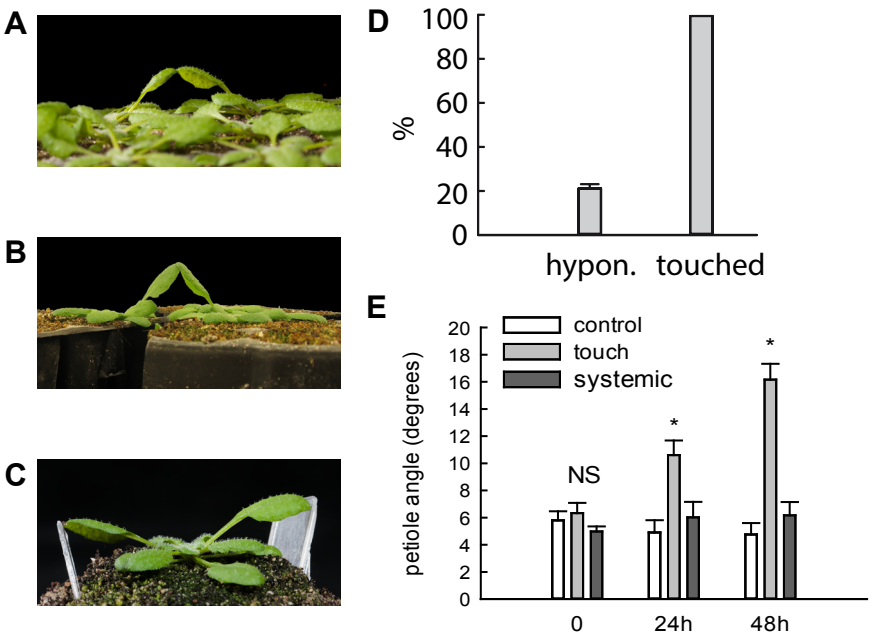
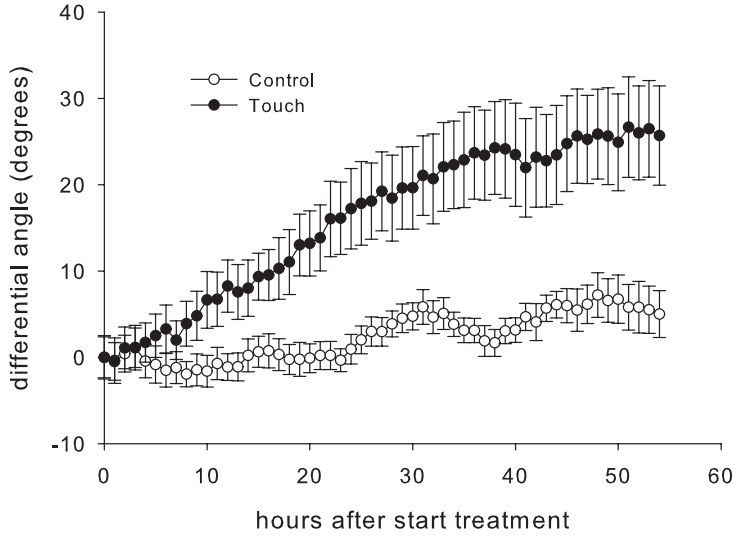


Figure 2.4 Touch-induced hyponasty. Representative photographs of leaves hyponastic through touch in (A) canopy-grown plants, (B) two single-grown plants with touching leaf tips, and (C) a single-grown plant touching a transparent tag. (D) Percentage of total leaves in canopy that is hyponastic, and percentage of hyponastic leaves that is being touched by other leaves. (E) Petiole angles after 24 h and 48 h from leaves of plants touching a transparent tag (touch) and untouched leaves from the same plants (systemic) or control plants (n = 10). Data represent means ± SE. Per time-point, data were tested for differences with a one-way ANOVA (asterisk represents significant difference from control, Bonferroni post-hoc, $P < 0.01$).

Figure 2.5 Time lapse of differential angle with start of the experiment (tn – t0) for control plants and plants touching a tag in continuous light. Data represent means ± SE (n = 10).



Mechanostimulation has been associated with the induction of so called *TOUCH* genes (Braam, 2005). We tested whether these genes are also induced in the touch treatments with tags but found no significant induction of these genes in either the petioles or leaf laminas (fig. 2.6 A and B). Furthermore, the microarray data from canopy plants at LAI 1.0 did not point toward a known regulatory pathway for touch responses (table S2.2). Gene ontology (GO) analysis showed an overrepresentation of several defence-related processes and brassinosteroid (BR) signalling, but not of mechanostimulation (table 2.1). The defence hormone jasmonic acid (JA) has recently been associated with touch-induced morphogenesis (Chehab et al., 2012). However, the JA mutants *coi1-1* and *jar1-1* showed a wild-type response to continuous touching, as did mutants in various other hormonal pathways and shade-avoidance mutants (fig. 2.7). The BR signalling mutant *bri1-1* seemed to show a somewhat reduced response, although it was still able to show touch-induced hyponasty (fig. 2.7D).

Taken together, these data show that mild, continuous physical touching of leaves can explain the early R:FR-independent hyponasty in Arabidopsis canopies. This suggests that touch is an important early neighbour detection signal that precedes the R:FR signal in dense stands of rosette species.

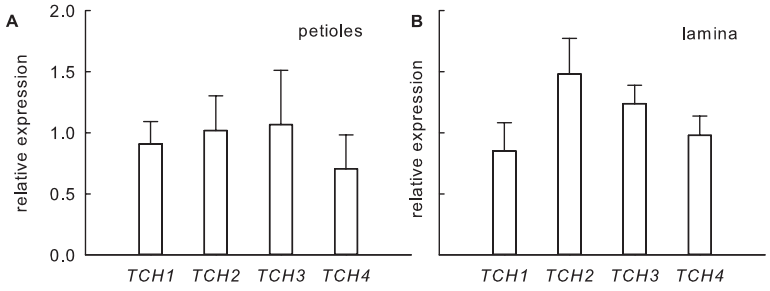


Figure 2.6 Touch-induced hyponasty does not involve induction of *TOUCH* genes. Relative expression of 4 *TOUCH* genes in petioles (A) and laminas (B) of leaves after 24 h of touching a transparent tag. Expression is given relative to non-touched control. UBQ5 was used as an internal standard. Data represent means ± SE (n = 4).

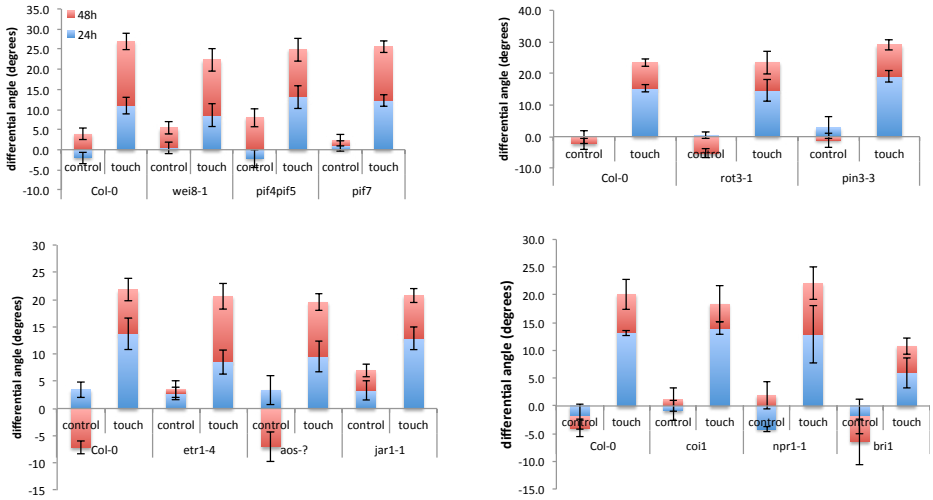


Figure 2.7 Touch-induced hyponasty is not regulated through a known touch mechanism. Differential angle with starting angle after 24 h and 48 h in leaves touching a tag in wild-type (Col-0) and shade-avoidance mutants (A); a brassinosteroid (BR) and auxin mutant (B); an ethylene and jasmonic acid mutants (C); and a jasmonic acid, a salicylic acid, and a BR mutant (D). Data represent means ± SE (n = at least 8).

Hyponasty is required to generate low R:FR conditions in vegetative Arabidopsis canopies

Next, we studied the functionality of the early hyponastic response in the Arabidopsis canopies. Elevating the leaves prior to the induction of elongation would ensure that growth is directed in a mostly vertical direction toward the light. We, therefore, tested whether hyponasty is a prerequisite for low R:FR-induced petiole elongation. We found that is not the case, since petioles of single-grown plants that were fixed in a horizontal position still elongated upon low R:FR (fig. 2.8).

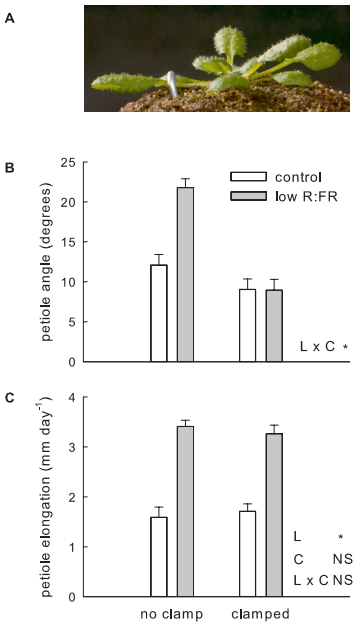


Figure 2.8 Shade-avoidance response to low R:FR in petioles fixed to the horizontal. (A) Photograph of a petiole clamped to the horizontal. (B) Petiole angles after 6 h and (C) petiole elongation after 24 h of petioles without clamp (no clamp) or fixed to the horizontal (clamped) in control light and low R:FR. Data represent means ± SE (n = 8). Asterisk represents significant difference (two-way ANOVA, $P < 0.05$). C, clamp treatment; L, light treatment; L:C, interaction between light and clamp treatment; NS, not significant.

Alternatively, we hypothesized that the touch-induced changes in canopy structure from the predominantly flat architecture of rosette plants toward a more vertical canopy orientation might increase the horizontal FR reflection by the elevated leaves toward neighbouring plants. We used a 3D virtual plant model based on phenotypic plant parameters from true canopies (fig. 2.1) at different LAIs and reflectance properties (Evers et al., 2007) to virtually increase the leaf angles at fixed LAI values and study if indeed the vertical leaf orientation affects the R:FR within the canopy. The model reproduced realistic virtual representations of the canopy and simulated the R:FR on all leaves individually, confirming the relatively small decrease in R:FR when hyponasty was first observed at LAI 0.65 (fig. 2.9 and fig. S2.3). We then manipulated the virtual leaf angles at LAI 0.65 to test the effect of leaf angles on the R:FR reflected onto neighbouring leaves. These simulations showed that high petiole angles that create a vertical canopy arrangement can bring down the R:FR in the canopy by ~50% (fig. 2.10). These data indicate that a decrease in the R:FR in dense stands of rosette plants depends on a hyponastic orientation of the leaves.

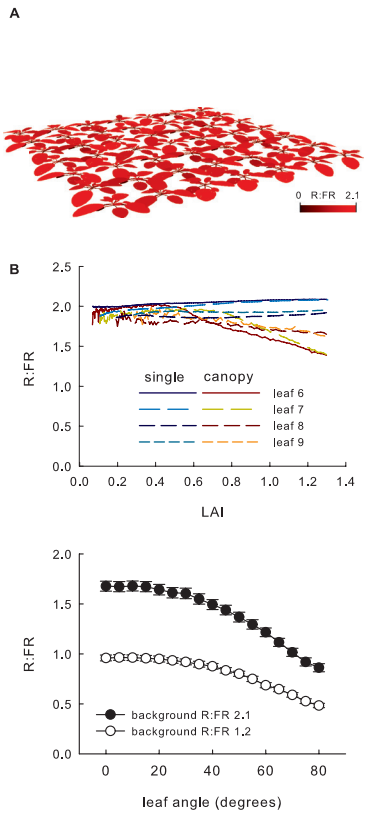


Figure 2.9 Simulations of a developing Arabidopsis canopy. (A) Virtual representation of a canopy at LAI 0.65. The brightness of the color corresponds to the R:FR on the leaves. (B) R:FR output of virtual canopy model for lamina of leaves 6–9 from canopy-grown and single-grown plants. These are the most responsive leaves and include the leaf measured in fig.1 (leaf 8). Data for single plants are plotted against canopy LAI and represent plants of the same age as their equivalent in the canopy at a specific LAI.

Figure 2.10 Average R:FR output for all laminas of a virtual canopy plant at LAI = 0.65 with manipulated leaf angles in background light with R:FR 2.1 or 1.2. Data represent means ± SE from nine simulated inner canopy or single-grown plants.

In natural light conditions, the R:FR ratio is usually around 1.2. Since we demonstrated in figure 2A that a reduction in R:FR from 2.1 to 1.2 induced hyponasty, we investigated the hyponastic response at natural light conditions. The virtual canopy model allowed us to study the reduction in R:FR under natural R:FR conditions by using a light source with R:FR = 1.2, representing natural sunlight as input (Smith, 2000), (fig. 2.11 A). The computed R:FR at LAI = 0.65 was found to be 1.0 (fig. 2.11 A). A reduction in R:FR from 2.1 to 0.9 resulted in an increased petiole angle, but a reduction in R:FR from 1.2 to 0.9 did not induce hyponasty. Petiole angles for plants grown at R:FR 2.1 were similar to the angles of plants grown at R:FR 1.2 (fig. 2.11 B). Thus, the hyponastic response to lowered R:FR ratios is at least partially dependent on the original R:FR. Previously, the R:FR has been described to decrease to ~0.6 at an LAI of 0.6 in stands of two stem-forming species in natural sunlight (Ballaré et al., 1990). In the simulated Arabidopsis canopies under natural light conditions, the petiole angles had to be virtually manipulated to >65° to reach this R:FR at LAI 0.65 (fig. 2.10).

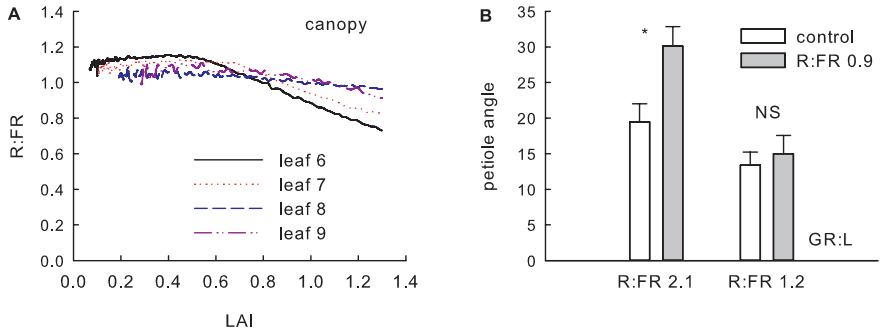


Figure 2.11 R:FR output of virtual canopy model for leaves 6–9 from canopy-grown plants in a background R:FR of 1.2 plotted against canopy LAI. The model output in A predicted that for canopy plants grown in background R:FR of 1.2, the R:FR at LAI =0.65 would be ~1.0. To test if this decrease in R:FR could induce hyponasty, plants were grown in control white light with R:FR 2.1 or R:FR 1.2 (obtained by mild FR supplementation) and subsequently transferred to treatment conditions that were either the same (controls) or had a reduced R:FR of 0.9 (supplemental FR) (B). Leaf angles after 6 h of treatment are shown. Data represent means ± SE (n = 10). Asterisk indicates significant difference (two-way ANOVA, $P < 0.05$). GR, growth R:FR ratio (grown at R:FR 2.1 vs. R:FR 1.2); L, light treatment (control vs. R:FR 0.9); GR:L, interaction between light treatment and growth ratio.

Touch is not priming for an enhanced FR response

Since touch-induced hyponasty is required to generate a low R:FR signal in dense Arabidopsis stands, we studied whether touch treatment would also affect subsequent low R:FR-induced petiole elongation. Before being transferred to low R:FR conditions, plants received a 72 h touch treatment, which continued during low R:FR exposure. Petiole lengths were measured before and after low R:FR exposure. After 24 h low R:FR petiole lengths of both control plants and touch treated plants were significantly increased, but no interaction between touch pre-treatment and low R:FR exposure was observed (fig. 2.12).

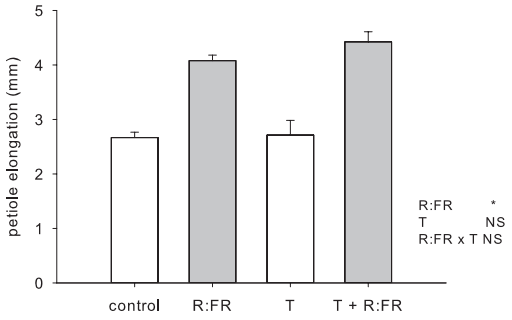


Figure 2.12 Average petiole length of untreated (control), low R:FR grown (R:FR), touched (T) and plants that receive a low R:FR and touch double treatment (T + R:FR). Petiole elongation was measured over 24 h, after 72 h touch pre-treatment. Data represent means (n = 10) ± SE. Significant low R:FR and touch effects were observed, no interaction between these factors was found (two-way ANOVA, $P > 0.05$).

Discussion

In dense vegetation, it is crucial for plants to perceive early signals from surrounding vegetation in order to respond timely and adequately to imminent shading. Small delays or reductions in shade avoidance responses can drastically reduce the competitive performance of individuals in dense stands (e.g. Pierik et al., 2003; Keuskamp et al., 2010), because of the asymmetric nature of competition for light (Weiner, 1990) . A decrease in R:FR has long been established as the earliest cue to sense the presence of proximate neighbours, because it can be perceived through light reflected from neighbouring leaves and thus constitutes a cue before actual shading takes place. Here, we provide evidence for leaf touching as a neighbour detection mechanism in competition for light that induces hyponastic leaf movement. Furthermore, we show that this touch-induced hyponasty is necessary to generate a low R:FR signal in a dense stand of rosette plants, characterized by horizontally growing leaves. Hyponastic growth in dense *A. thaliana* stands occurred well before enhanced petiole elongation in response to neighbouring plants (fig. 2.1). These response kinetics resemble the submergence response of the semiaquatic plant *Rumex palustris* (Pierik et al., 2011). Enhanced petiole elongation during submergence in this species occurs only when an angle of 40–50° was reached (Cox et al., 2003), thereby providing the plant with a mechanism to direct its growth investment such that it contributes to surviving the submergence stress by restoring aerial contact of the shoots. Different from the submergence example, low R:FR-induced petiole elongation still occurred when petioles were kept horizontally (fig. 2.8) and, therefore, does not depend on an elevated leaf angle.

However, a computational modeling approach of our canopy setup showed that more vertically oriented hyponastic leaves are essential to create a low R:FR signal in the absence of truly overlapping and thus shading leaves, thereby presenting hyponastic leaf growth in canopies to be required for subsequent petiole elongation in the rosette species Arabidopsis. This also explains why in their seminal paper on early neighbour detection, Ballaré et al. (1990) found R:FR reductions in dense stands of the two stem-forming species *Datura ferox* (fierce thornapple) and *Sinapis alba* (white mustard) that through modeling could only be approached in the Arabidopsis canopies at a similar LAI when petiole angles were virtually manipulated to ~65°. This finding shows the importance of a vertical growth structure for the generation of a low R:FR signal that can be perceived by neighbours. Such height growth is achieved in vegetative rosette canopies by neighbour-induced hyponastic growth. The early, R:FR-independent hyponastic response observed here in dense Arabi-

dopsis stands appears to be induced by touching neighbouring leaves. Although early changes in the R:FR within a canopy are mainly caused by touching leaves, the touch response itself did not affect low R:FR enhanced petiole elongation (fig. 2.12), suggesting that low R:FR induced petiole elongation acts independently from touch induced hyponasty. Responses to touch or to mechanical stimulation in general are called thigmomorphogenic responses and typically include strengthening of the exposed tissue by decreased elongation growth and increased diameter (as reviewed in Coutand, 2010). Mechanostimulation of plant tissues is associated with the induction of *TOUCH* (*TCH1-4*) genes that encode different physiological regulators such as the XTH22 protein and calmodulins (Braam, 2005). Interestingly, we found no induction of the four main *TCH* genes in the mild treatment that mimicked touch from a neighbouring plant (fig. 6), implying that touch-induced hyponasty is not controlled through the established thigmomorphogenic pathways.

Recently, the hormone jasmonic acid (JA), a well-known regulator of plant defences (e.g. Pieterse et al., 2012, Chapter 4), has been implicated in thigmomorphogenesis (Chehab et al., 2012), but JA mutants showed a wild-type hyponastic response to continuous touch (fig. 2.7 C & D). Because the situation in dense stands with two leaves pushing each other upward is particular in that the mechanical force is mild, comes from both sides, and increases with time rather than being static, it seems plausible that at least parts of the regulatory pathway are different from established touch-signalling components and thus remain to be identified in future studies. The fact that the touch-induced response described here is regulated differently from the better-known mechanical stimulation responses would also be key to competitive success, because the classic response to mechanical stimulation of decreased elongation growth would reduce competitive performance in dense stands (Anten et al., 2005). Some examples of growth induction by mechanical stimulation, other than touch during competition, have been described previously. For example, hypocotyl elongation can be stimulated by mild vibration in seedlings of various species, including Arabidopsis (Johnson et al., 1998; Takahashi et al., 1991). This finding shows that a very light touch could indeed positively affect cell elongation, which is also driving hyponastic growth (Polko et al., 2012), although the mechanisms through which growth is stimulated remain to be elucidated. Our microarray data indicated that BR may be involved (table S2), and the BR signalling mutant *bri1-1* indeed seemed to show a somewhat reduced hyponastic response to touch (fig. 2.7 D). This may serve as a starting point for future studies, also because brassinosteroids have recently been associated with hyponastic growth in response to ethylene (Polko, 2012).

We conclude that the earliest response to neighbours in dense stands of Arabidopsis is induced by touching of leaves. Thigmomorphogenesis is likely to occur in most dense stands of wild plants and crops, but touch-induced neighbour responses might be a particularly important signal for stands of horizontally growing rosette plants that generate less FR reflection than stem-forming plants at an early stage of competition.

Acknowledgements

We thank Ronald Leito, Martijn van Zanten, Rob Welschen, and Lot Gommers for technical assistance. This research was funded by The Netherlands Organization for Scientific Research (NWO) Grants 021.001.030 (to M.d.W.) and 818.01.003 (to R.P. and W.K.).

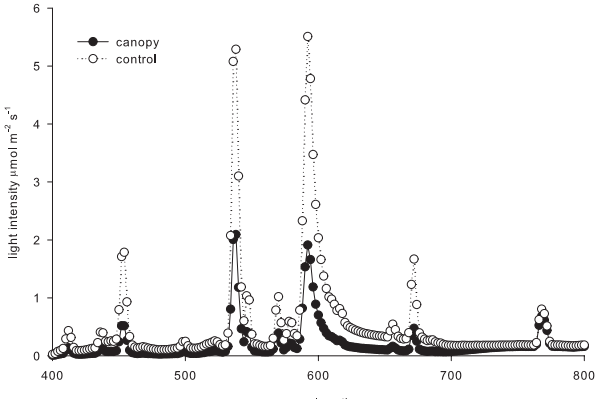


Figure S2.1 Light spectrum of control light (white symbols) and canopy environment at LAI 2.54 (black symbols).

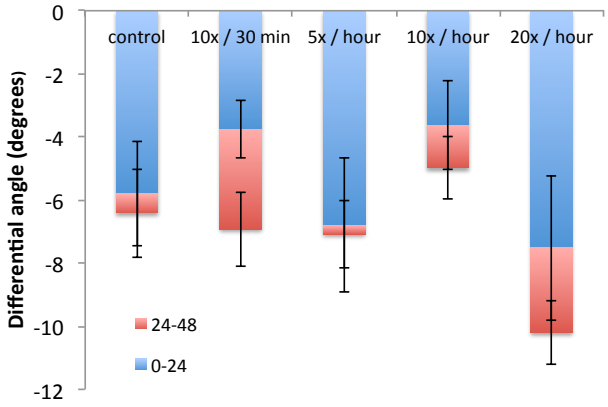


Figure S2.2 Differential petiole angles after 24 h and 48 h from leaves that were lightly touched with a brush at the leaf tip in series with different intervals and different frequencies. Touch treatments varied in the number of times a plant was touched per time point and the frequency of repetition. Plants were either untouched, touched 10 times every 30 minutes or touched 5/10/20 times every hour during the light period. Data represent means ± SE (n = 10).

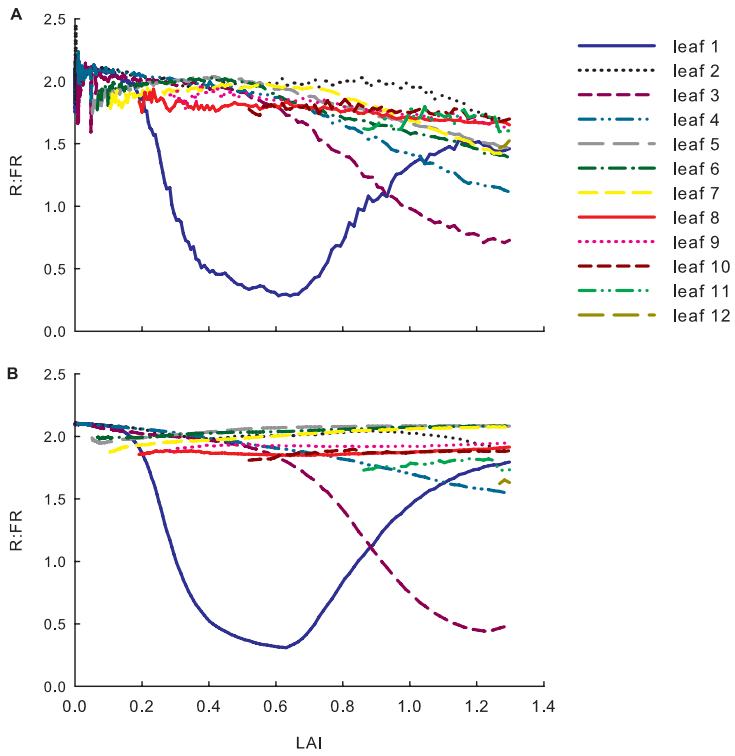


Figure S2.3 Simulations of a developing Arabidopsis canopy. Model output of R:FR for all leaf laminas of canopy (C) and single (D) plants over time plotted as LAI at background R:FR 2.1. Data for single plants (D) are plotted against canopy LAI and represent plants of the same age as their equivalent in the canopy at a specific LAI. Data are means from nine simulated plants.

Gene	Primer	Sequence
ATHB2	F	GAGGTAGACTGCGAGTTCTTACG
	R	GCATGTAGAACTGAGGAGAGAGC
XTH15	F	CGGCACCGTCACTGCTTAC
	R	GAAACTCAAAGTCTATCTCGTCATGTG
UBQ5	F	CCAAGCCGAAGAAGATCAAG
	R	ACTCCTTCCTCAAACGCTGA
TCH1	F	TTCATCCTCCCTTTCCCTCT
	R	AGCAGCTGAGATGAAACCGT
TCH2	F	CCAACAGCATCACCAGAAGA
	R	CACAGAGCACTTCTCACCCA
TCH3	F	TCCAGGACATGATCAACGAA
	R	ACAGCGCTTCGAACAAATCT
TCH4	F	CTACTGGCTCGTGGTTGTCA
	R	CCTCTTCGCATCCGTACAAT

Table S2.1 Primer sequences used for real-time RT-PCR

Table S2.2 Differentially expressed genes with cut-off value of B>2 in petioles from canopy-grown plants at LAI 1.0

locus	description
AT1G14870; AT1G14880	PLANT CADMIUM RESISTANCE 2; AT1G14880, PLANT CADMIUM RESISTANCE 1
AT3G60420	Phosphoglycerate mutase family protein
AT4G14365	XB3 ortholog 4 in Arabidopsis thaliana
AT5G24210	alpha/beta-Hydrolases superfamily protein
AT3G01290	SPFH/Band 7/PHB domain-containing membrane-associated protein family
AT5G10760	Eukaryotic aspartyl protease family protein
AT5G24530	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT4G33050	calmodulin-binding family protein
AT3G50480	homolog of RPW8 4
AT5G09220	amino acid permease 2
AT2G26440	Plant invertase/pectin methylesterase inhibitor superfamily
AT2G31880	Leucine-rich repeat protein kinase family protein
AT3G19010	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT3G57260	beta-1,3-glucanase 2
AT5G54610	ankyrin
AT4G38550	Arabidopsis phospholipase-like protein (PEARLI 4) family
AT3G45860	cysteine-rich RLK (RECEPTOR-like protein kinase) 4
AT3G19710	branched-chain aminotransferase4
AT1G23130	Polyketide cyclase/dehydrase and lipid transport superfamily protein
AT4G39950	cytochrome P450, family 79, subfamily B, polypeptide 2
AT1G75040	pathogenesis-related gene 5
AT4G13180	NAD(P)-binding Rossmann-fold superfamily protein
AT2G37710	receptor lectin kinase
AT3G45640	mitogen-activated protein kinase 3
AT2G46600	Calcium-binding EF-hand family protein
AT3G49620	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT2G46440; AT2G46430	cyclic nucleotide-gated channels; cyclic nucleotide gated channel 3
AT2G32160	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
AT1G03400	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT3G26210	cytochrome P450, family 71, subfamily B, polypeptide 23
AT1G55910	zinc transporter 11 precursor
AT1G75750	GAST1 protein homolog 1
AT1G32450	nitrate transporter 1.5
AT3G14990	Class I glutamine amidotransferase-like superfamily protein
AT2G17040	NAC domain containing protein 36
AT3G14620	cytochrome P450, family 72, subfamily A, polypeptide 8
AT4G36500	unknown protein
AT3G48720	HXXXD-type acyl-transferase family protein
AT4G08950	calreticulin 3
AT2G23680	Cold acclimation protein WCOR413 family

Table S2.2 continued

locus	description
AT4G00780	TRAF-like family protein
AT1G67860	unknown protein
AT3G48990	AMP-dependent synthetase and ligase family protein
AT5G52810	NAD(P)-binding Rossmann-fold superfamily protein
AT4G23180	cysteine-rich RLK (RECEPTOR-like protein kinase) 10
AT2G13790; AT2G13800	somatic embryogenesis receptor-like kinase 4; somatic embryogenesis receptor-like kinase 5
AT5G17760	P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT1G80130	Tetratricopeptide repeat (TPR)-like superfamily protein
AT1G26380	FAD-binding Berberine family protein
AT3G45620	Transducin/WD40 repeat-like superfamily protein
AT1G76240	Arabidopsis protein of unknown function (DUF241)
AT1G71040	Cupredoxin superfamily protein
AT1G31130	unknown protein
AT3G11964	RNA binding;RNA binding
AT1G80050	adenine phosphoribosyl transferase 2
AT3G16920	chitinase-like protein 2
AT1G74030	enolase 1
AT5G45040	Cytochrome c
AT5G61130	plasmodesmata callose-binding protein 1
AT5G05160	Leucine-rich repeat protein kinase family protein
AT4G33420	Peroxidase superfamily protein
AT3G62020	germin-like protein 10
AT1G24020	MLP-like protein 423
AT5G27450	mevalonate kinase
AT4G02290	glycosyl hydrolase 9B13
AT1G10522	unknown protein
AT4G34160	CYCLIN D3;1
AT4G02850	phenazine biosynthesis PhzC/PhzF family protein
AT2G36570	Leucine-rich repeat protein kinase family protein
AT2G43800	Actin-binding FH2 (formin homology 2) family protein
AT5G07030	Eukaryotic aspartyl protease family protein
AT5G64080	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT1G28290	arabinogalactan protein 31
AT4G08950	Phosphate-responsive 1 family protein
AT2G38080	Laccase/Diphenol oxidase family protein
AT2G33850	unknown protein
AT4G08950	Pectin lyase-like superfamily protein
AT5G57560	Xyloglucan endotransglucosylase/hydrolase family protein

CHAPTER

1 2 3 4 5 6

Ethylene modulates shade avoidance responses to low Red : Far-red light conditions in *Arabidopsis thaliana*

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Abstract

Plants rely on a variety of cues from their environment, including those that help them detect competing vegetation. Perception of ethylene and changes in light quality are established cues that play important roles in plant-plant interactions. Ethylene has been suggested to function both as a cue to detect neighbours and as a component in the light quality-mediated signal transduction pathway that leads to shade avoidance responses. The predominant light quality signal that occurs between competing plants in dense stands is a reduced red : far-red light ratio (R:FR) caused by absorption of red light and reflection of far-red light by proximate plants. Here, we investigate to which extent ethylene perception and production are required for a complete response to low R:FR conditions in *Arabidopsis thaliana*. We demonstrate that ethylene emissions are strongly enhanced in low R:FR conditions. Ethylene insensitive plants respond less accurately to strong reductions in R:FR and do not show enhanced petiole elongation in light environments with only a modest reduction in R:FR, such as also occurs in early phases of canopy development. We conclude that ethylene modulates shade avoidance responses to a reduction in R:FR, by enhancing the plant's sensitivity to subtle changes in R:FR and allowing a plant to respond faster to strong reductions in R:FR.

Introduction

In dense stands, plants compete above and belowground for resources such as light, water and nutrients. As photoautotrophic organisms, plants depend strongly on interception of photosynthetically active radiation (PAR, λ = 400-700 nm). In dense stands, small differences in size can

lead to major effects on light capture and thus plant fitness (e.g. Pierik et al., 2003; Schmitt et al., 1995). Shade-intolerant plants have evolved a suite of phenotypic responses, defined as the shade avoidance syndrome, to avoid shading by neighbouring plants. In order to detect neighbours, plants make use of a range of cues, such as a reduction in the ratio between red (R) and far-red (FR) light (reviewed in Ballaré, 2009; Franklin, 2008), blue light fluence rates (reviewed in Ballaré, 1999; Vandenbussche et al., 2005), touching leaf tips from neighbouring plants (Chapter 2) or perception of the volatile hormone ethylene (Pierik et al., 2003).

Upon perception of a reduced R:FR ratio, plants typically induce shade avoidance responses that help to enhance light capture and thus increase their fitness (Callaway et al., 2003; Violle et al., 2009). These responses include a more vertical orientation of leaves, stem elongation and apical dominance (reviewed by Franklin, 2008). In addition to these morphological changes, plant exposure to low R:FR leads to an increase in the production of the volatile plant hormone ethylene in *Arabidopsis thaliana*, *Nicotiana tabacum*, *Pisum sativum* and *Sorghum bicolor* (Pierik et al., 2009; Pierik et al., 2004; Finlayson et al., 1999; Foo et al., 2006). However, ethylene production is not increased in all plant species upon exposure to low R:FR. For example, shoots of *Stellaria longipes* do not produce more ethylene under low R:FR conditions (Kurepin et al., 2006b) and in *Helianthus annuus* low R:FR exposure stimulates ethylene production in leaves, but reduces emission in internodes (Kurepin et al., 2007). Exposure to ethylene can affect plant growth in a dose-dependent manner and both growth promotion and inhibition have been described (reviewed in Pierik et al., 2006). Furthermore, ethylene strongly promotes a vertical leaf orientation through hyponastic petiole growth in shade-avoiding species such as Arabidopsis (Millenaar et al., 2005) and tobacco (Pierik et al., 2004), but also in flood tolerant plants such as *Rumex palustris* (Cox et al., 2003).

Consistent with these observations it was found that ethylene perception is required for successful aboveground competition with neighbouring plants in tobacco (Pierik et al., 2004). Pierik et al (2004) showed that wild type and ethylene-insensitive transgenic plants performed similarly when grown in a high-density monoculture. However, when ethylene insensitive tobacco plants were grown in a high-density mixture with wild type plants, the mutants were outcompeted by their neighbours. The ethylene-insensitive plants did not respond fast enough with enhanced petiole elongation and hyponasty to reduced PAR and blue light, but were still able to respond to changes in R:FR conditions (Pierik et al., 2004a). Furthermore, ethylene levels in the canopy atmosphere of dense, greenhouse-grown tobacco stands were found to be elevated to functional levels (Pierik et al., 2004).

In this chapter, the role of ethylene in high density and low R:FR induced shade avoidance responses is investigated in Arabidopsis. We show that in dense stands, ethylene emission is not induced by touching leaf tips. Furthermore, we demonstrate that ethylene-insensitive mutants display a disturbed response to a reduced R:FR ratio relative to wild type plants.

We conclude that ethylene is involved in the modulation of low R:FR induced hyponasty and petiole elongation. This modulating role can be of major importance in competition for light, because changes in R:FR conditions in dense stands are often gradual (chapter 2) and small differences in initial responses can determine the competitive success of individuals.

Materials and Methods

Plant growth and measurements

Wild-type Columbia-0 (Col-0) and the *ein2-1* and *etr1-4* ethylene-insensitive mutants in Col-0 background (Alonso et al., 1999; Bleecker et al., 1988) were stratified (dark, 4 °C, 3 d) and subsequently grown on soil. Plant growth and climate chamber settings were identical to the settings described in chapter 2. Analysis of plants grown in canopies and touch experiments were performed as described in chapter 2.

Chemical inhibitors

To inhibit ethylene biosynthesis, plants were soil-drenched with 30 ml amino-ethoxy-vinylglycine (AVG) in different concentrations (1, 5 or 10 μM AVG), 18h before start of the light treatment. AVG blocks ethylene biosynthesis by inhibiting the rate-limiting enzyme ACC synthase, thereby limiting the production of 1-aminocyclopropane-1-carboxylic-acid (ACC), the immediate precursor of ethylene (Amrhein & Wenker, 1979). Before AVG-application, plants were not supplied with water for 4d to maximize uptake. To inhibit ethylene perception, plants were gassed with 2 ppm 1-methyl cyclopropane (1-MCP) in air for 2h. 1-MCP completely blocks ethylene binding to the ethylene receptors (Sisler & Serek, 2003). To obtain a concentration of 2 ppm, 10 mg 0.14% Smartfresh (Rohm & Haas, Philadelphia, PA, USA) was dissolved in 1 ml water. Gas was collected for 15 minutes in a syringe and subsequently injected into glass jars (22.4 l) in which the plants were placed.

Light manipulations

R:FR ratio manipulations occurred through supplemental FR LEDs (730 nm; Philips Green Power) in a control white-light background of 110 μmol m–2 s–1 photosynthetically active radiation (PAR). Control plants were exposed to the same light intensity without additional FR light.

Ethylene production

Ethylene production was measured on freshly harvested shoot tissue (~1.0-1.2 g fresh weight) after 20 min of headspace accumulation in a syringe, as described previously (Millenaar et al., 2005), using the GC955 gas chromatograph with Photo ionisation detector and 160 cm Haye Sep R column, filled with Haye Sep 80/100 mesh (Synspec, Groningen, The Netherlands).

Statistics

Data were analysed through ANOVA or Student's *t* test in the IBM SPSS statistics 20 software.

Results

Ethylene control of shade avoidance in dense stands

The data in chapter 2 demonstrate that neighbour detection by touching leaf tips precedes physiologically meaningful changes in R:FR light conditions. To investigate whether ethylene might mediate this early touch-mediated neighbour response, ethylene emission from canopy plants was measured. As shown in fig. 3.1 A, canopy plants produced more ethylene than single-grown plants when the leaf area index (LAI; leaf area per soil area) was 0.6 or higher. However, at this LAI no elevated ethylene concentrations in the air between plants in these Arabidopsis canopies could be detected (data not shown). Ethylene emission by plants with leaves growing against a transparent tag (touch treatment) was similar to ethylene emission by control plants (fig. 3.1 B).

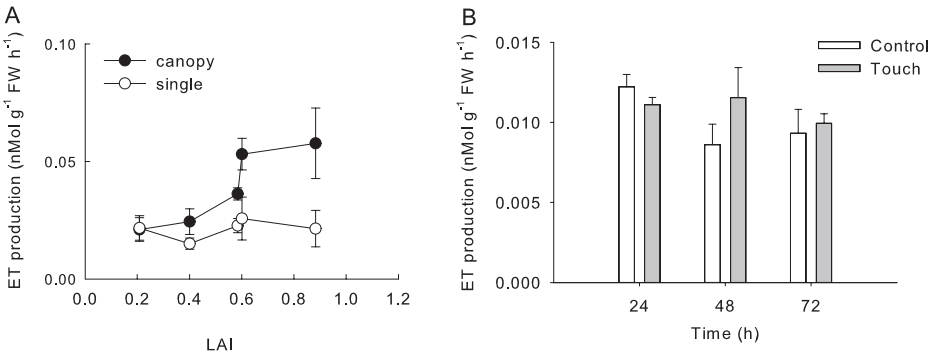

Figure 3.1 Ethylene production in response to neighbours and light quality. A) ethylene (ET) production in canopy-grown (filled symbols) and single-grown plants (open symbols) over time (n = 4). ET emission is significantly induced in canopy plants from LAI = 0.40 (Student's t test, $P < 0.05$). B) ET production of single-grown plants touching transparent tags over time (N=5), no significant differences were detected (Student's t-test per time point, $P>0.05$).

Effects of a strong reduction in R:FR (from 2.1 to 0.2) have been shown to affect petiole elongation, hyponasty and ethylene production (Franklin, 2008; Pierik et al., 2004a, b). However, in dense stands, the different neighbour detection signals change with time from weak signal intensity at early phases of development to strong signal intensity in well-developed stands (e.g. Chapter 2). To address these dynamics, ethylene production, petiole elongation and hyponasty of plants grown at different R:FR ratios were assessed. In fig. 3.2, effects of a mild reduction in R:FR on wild type plants is shown. Ethylene production, petiole elongation and hyponasty were induced by a reduction of R:FR from 2.0 (control conditions) to 0.2 (fig. 3.2). However, a reduction in R:FR from 2.0 to 1.5 did not lead to changes in ethylene production, hyponasty or petiole elongation. Differences between control and low R:FR grown plants were more pronounced in petiole angles than differences in petiole angles for plants exposed to R:FR 1.2 and 0.9.

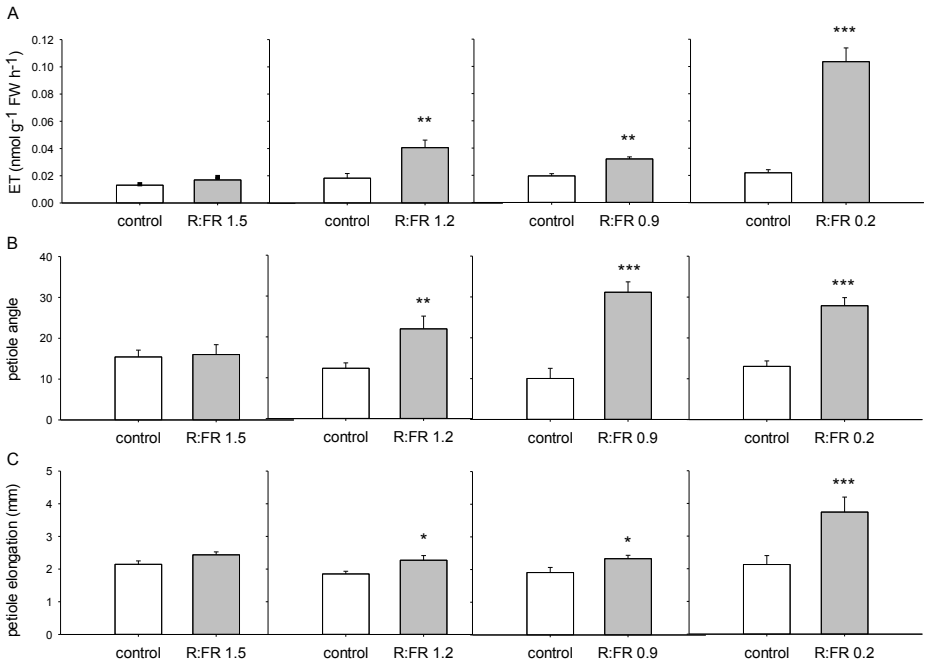

Figure 3.2 Ethylene (ET) production (A-D), petiole angle (E-H) and petiole elongation (I-L) of plants grown under control light with R:FR = 2.1 (white bars) and reduced R:FR (grey bars). Data represent means ± SE (n=5 for ET production measurements, n=10 for petiole angle and petiole elongation). Data were analysed with a Student's t-test. Significant differences are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

To investigate whether perception of elevated ethylene emissions is used as a cue to detect neighbours or is required to induce hyponasty and petiole elongation in reduced R:FR conditions, canopy development for wild type and ethylene-insensitive mutants (*etr1-4*) was monitored. The *etr1-4* mutant did not show enhanced petiole elongation in dense stands at LAI 1.1, while wild type plants did (fig. 3.3 A). The petiole angles of *etr1-4* mutants, on the other hand, were similar to wild-type plants, both in canopy and single-grown plants (fig. 3.3 B). To test whether differences in petiole elongation between Col-0 and *etr1-4* in high density were due to responsiveness to reductions in R:FR, the two genotypes were exposed to an R:FR ratio of 0.8. After 24h of exposure petiole elongation of Col-0 wild type was enhanced, while no effect of a reduced R:FR on petiole growth of *etr1-4* was found (fig. 3.4), indication that *etr1-4* is less sensitive for changes in R:FR conditions than Col-0.

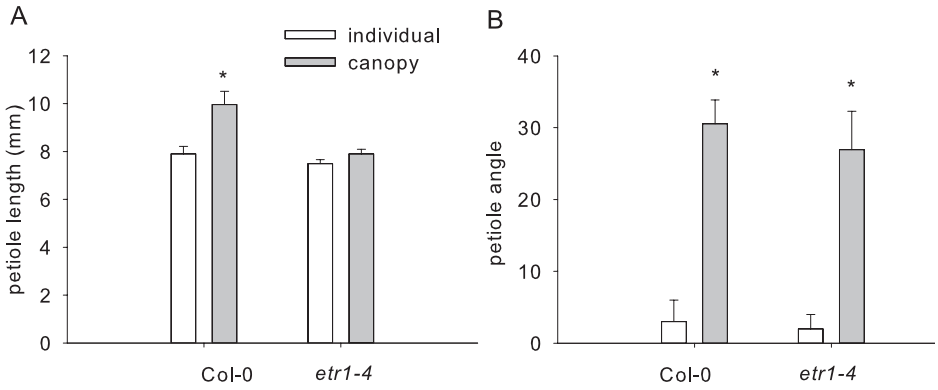

Figure 3.3 Petiole length (A) and angle (B) of the third-youngest leaves of single-grown (white bars) and canopy-grown (grey bars) plants (Data represent means ± SE, n = 9, canopy measurements were taken in two independent canopies). Data were analysed with a Student's *t*-test. Significant differences are indicated by * (P<0.05) or *** (P<0.001).

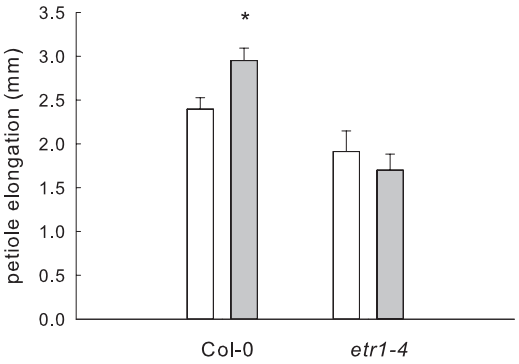

Figure 3.4 Petiole elongation of wildtype Col-0 and ethylene-insensitive *etr1-4* plants under control light (RFR 2.1; white bars) and reduced R:FR (R:FR = 0.8; grey bars). Data represent means ± SE (n = 10). Asterisk represents significant difference (p<0.05).

The role of ethylene in low R:FR-induced shade avoidance

Ethylene emissions increased with decreasing R:FR and the highest emissions were recorded for the lowest R:FR tested, i.e. 0.2, which also induced the strongest petiole elongation response (Fig. 3.2). This raised the question whether the extent to which ethylene emissions are increased by low R:FR conditions determines the degree of petiole elongation. We manipulated ethylene production with a well-established inhibitor, aminoethoxyvinylglycine (AVG). A soil drench with 10 µM AVG indeed fully blocked the increased ethylene emission caused by low R:FR exposure, whilst leaving the base level emissions under control conditions more or less intact (fig. 3.5 A). However, these plants showed similar petiole elongation and hyponasty upon 24 h exposure to a strongly reduced R:FR of 0.2 as plants in which ethylene biosynthesis was not inhibited (fig. 3.5 B & C).

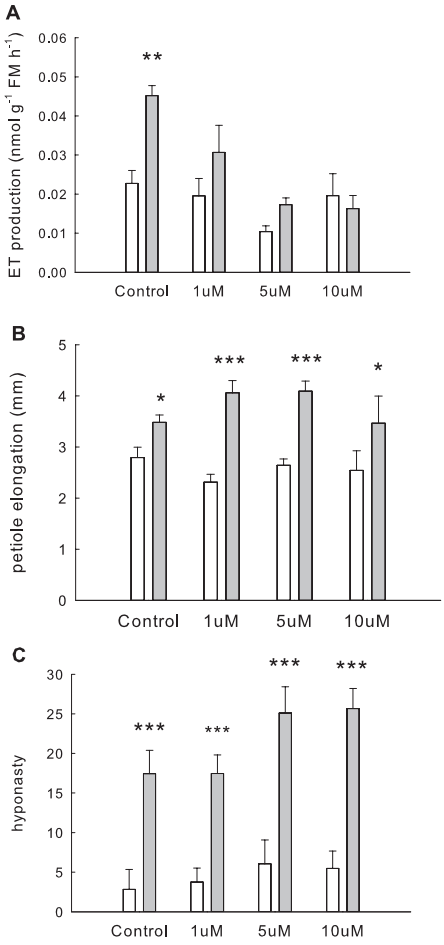

Figure 3.5 Ethylene (ET) production (A), petiole elongation (B) and hyponasty (C) of plants grown in control light (R:FR 2.1; white bars) and low R:FR (R:FR 0.2; grey bar). Plants were treated with different concentrations of aminoethoxyvinylglycine (AVG). Data represent means ± SE (n=5 for ET production measurements, n=10 for petiole angle and petiole elongation). Data were analysed with a Student's t-test. Significant differences are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

To verify whether ethylene signalling was important at all for shade avoidance responses to the severely reduced R:FR of 0.2, plants were treated with 1-methylcyclopropene (1-MCP), a potent inhibitor of ethylene perception (Sisler & Serek, 2003). Although 1-MCP-treated plants showed similar petiole elongation as untreated plants (fig. 3.6 A), low R:FR-induced hyponasty was not observed after 6h of low R:FR exposure (fig. 3.6 B. After 24h, no difference between the petiole angle of 1-MCP treated and untreated plants in low R:FR was observed (fig. 3.6 C). To confirm these observations, the ethylene-insensitive mutant *ein2-1* was studied. We found that *ein2-1* displayed a somewhat reduced low R:FR-induced petiole elongation response (fig. 3.6 D) and a delayed hyponastic response (fig. 3.6 E & F).

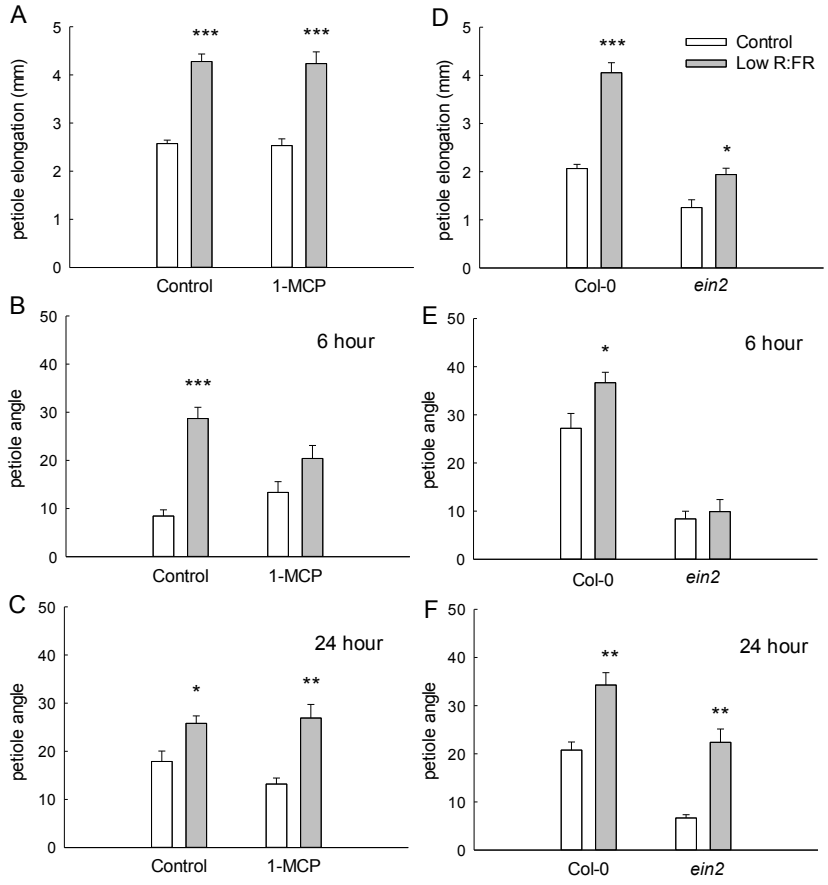

Figure 3.6 Ethylene (ET) production (A), petiole elongation (B) and hyponasty (C) of plants grown in control light (white bars) and reduced R:FR (grey bars, R:FR 0.2). Plants were either or not treated with 2 ppm 1-methylcyclopropene (1-MCP). Ethylene production (D), petiole elongation (E) and hyponasty (F) of wild type and ethylene insensitive *ein2-1* plants grown in control light (R:FR 2.1; white bars) and reduced R:FR (R:FR 0.2; grey bars). Data represent means ± SE (n=5 for ET production measurements, n=10 for petiole angle and petiole elongation). Data were analysed with a Student's t-test. Significant differences are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

To better understand the differences in hyponasty kinetics between wild type and *ein2-1*, plants were placed at R:FR 0.2 and photographed every 30min. As shown in fig. 3.7, petiole angles of wild type plants started to increase 4h after start of low R:FR treatment. After 7h, the petiole angle of those plants had increased by 25 degrees and reached the highest recorded petiole angle (32 degrees) to the horizontal. In *ein2-1*, petiole angles started to increase by just a few degrees after approximately 6 hours of low R:FR exposure, but remained low as compared to Col-0. After the night period, *ein2-1* angles in low R:FR had increased to approximately 22 degrees, which was still much lower than Col-0 (approx. 35 degrees) (fig. 3.7). Thus, the ethylene-insensitive mutant *ein2-1* indeed displays reduced hyponasty in low R:FR (0.2) as compared to wild type plants.

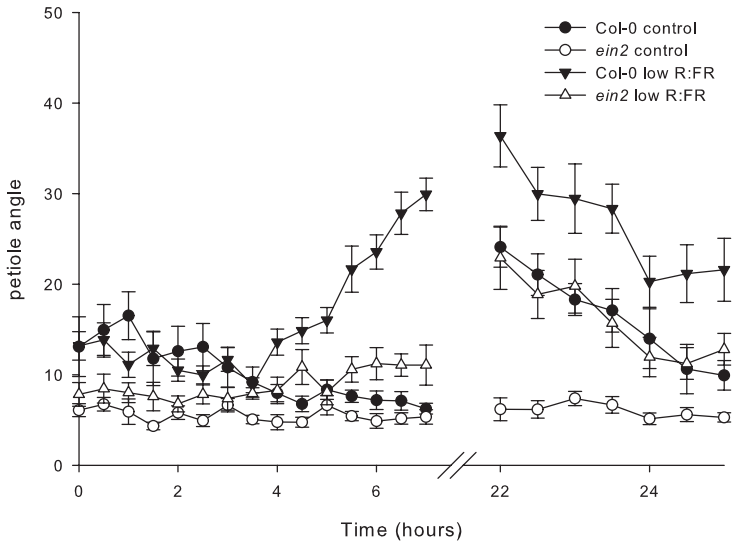

Figure 3.7 Petiole angles of plants grown in control light conditions (R:FR 2.1; circles) or low R:FR conditions (R:FR 0.2; triangles) of wild-type (filled symbols) and ET-insensitive mutant *ein2* (open symbols) over time (n = 10). Data represent means ± SE.

Discussion

We studied the role of ethylene in plant responses to neighbours in dense stands. Although we found that elevated ethylene emissions in dense stands are not related to touching leaf tips, we did find evidence for an involvement of ethylene in regulating responses to reduced R:FR ratios induced by neighbouring vegetation.

Early hyponasty is associated with touching leaf tips, but *etr1-4* shows a similar touch-response as wild type (chapter 2) and touch itself does not lead to increased ethylene emissions. Therefore, this early hyponastic response is most likely independent of ethylene signalling. It was argued in chapter 2 that the response to touching leaves of neighbouring leaves was regulated in a way that is different from the classical thigmomorphogenic responses. Ethylene has previously been associated with thigmomorphogenic responses in for example tobacco (Anten et al., 2006). However, mechanical stimulation in Arabidopsis has been shown to occur independently of ethylene signalling (Johnson et al., 1998; chapter 2), which was also observed in the current study.

Interestingly, the absence of petiole elongation in *etr1-4* in canopies at LAI 1.1 corresponds with the reduced ability of this mutant to respond to mild changes in reduced R:FR conditions, since a reduction in R:FR from 2.0 to 0.8 did not lead to enhanced petiole elongation in *etr1-4*, whereas it did enhance petiole elongation in wild type Col-0 (fig. 3.4). It thus appears that ethylene signalling is key to early neighbour-induced petiole elongation, most likely through involvement in the response to moderately reduced R:FR ratios that occur at relatively early stages of canopy development. Ethylene, however, does not seem to be a major regulator of petiole elongation responses to more severe R:FR reduction that occur in late phases of canopy development (figs. 3.5 & 3.6).

This is consistent with the reduced shade avoidance responses at early canopy stages in high-density grown tobacco, although in tobacco canopies leaf angles were also clearly reduced in ethylene-insensitive plants (Pierik et al., 2003). It is, however, unknown whether in these tobacco stands early hyponasty was caused by mild R:FR reduction, by touch as was found for Arabidopsis in Chapter 2, or by a combination of these cues.

Although *ein2-1* plants showed a reduced petiole elongation response even to strongly reduced R:FR conditions (from 2.1 to 0.2) that represent late stages of canopy development, Col-0 plants treated with 1-MCP showed a response that was identical to control plants. This discrepancy may be associated with putative pleiotropic effects of the *ein2-1* mutant, for example on ABA signalling (e.g. Beaudoin et al., 2000). The less severe leaf angle phenotype of 1-MCP treatment as compared to the *ein2-1* mutant could also imply less effective suppression of ethylene signalling by 1-MCP as compared to the genetic knockout. However, we verified whether 1-MCP treated Col-0 plants were still able to respond to exogenous ethylene and found that ethylene-induced hyponastic leaf growth was completely lost. Interestingly, the hyponastic response to low

R:FR (0.2) was delayed in both the ethylene-insensitive *ein2-1* mutant and 1-MCP-treated plants as compared to control wild type plants as was visible upon 6 h exposure to low R:FR conditions (figs. 3.5-3.7). After 24 h, however, petiole angles in low R:FR were similar between ethylene-insensitive and control plants, indicating that ethylene modulates the rate of the response, rather than the magnitude, which is reminiscent of what was observed previously in tobacco (Pierik et al., 2004b).

Interestingly, Pierik et al. (2009) previously demonstrated that several ethylene-insensitive mutants showed reduced low R:FR-induced petiole elongation, similar to what is found here for *ein2-1*, but different from the undisturbed petiole elongation response upon 1-MCP treatment (Fig. 3.6a). These differences may be associated with differences in plant developmental stage. Pierik et al. (2009) studied plants that were nearly in the bolting stage whereas much younger plants were used in the current study. It is possible that both shade avoidance and ethylene responses vary with developmental age. Accordingly, light-grown Arabidopsis seedlings show clear promotion of shoot elongation by ethylene ((Smalle et al., 1997; Pierik et al., 2009), whereas plants of the same species do not show pronounced shoot elongation responses at later stages of plant development (Millenaar et al., 2005). Similarly, enhanced ethylene production in *Nicotiana attenuata* as a response to wounding plus oral secretion of *Manduca sexta* is dependent on plant age: in (nearly) bolting plants, the wounding plus oral secretion-elicited levels of ethylene were lower than in plants that were still in their vegetative state (Diezel et al., 2011).

Auxin transport has been shown to regulate low R:FR-induced elongation responses (Keuskamp et al., 2010) and also ethylene-dependent elongation growth acts at least partially through auxin signalling and auxin transport (Stepanova et al., 2008; Pierik et al., 2009). In root elongation, ethylene stimulates both auxin biosynthesis and auxin transport (Ruzicka et al., 2007; Stepanova et al., 2007). As mentioned earlier, a reduction of the R:FR from 2.1 to 0.8 stimulates petiole elongation in an ethylene-dependent manner. It is possible that ethylene further enhances the auxin-dependent elongation response that is activated upon low R:FR exposure, which involves enhanced auxin biosynthesis (Tao et al., 2008) and lateral transport towards the outer cell layers of elongating organs (Keuskamp et al., 2010). We are, however, not aware of studies that have verified interactions between ethylene and auxin signalling under low R:FR conditions. Neither has it been researched whether these interactions control hyponastic leaf growth under low R:FR conditions.

It has, however, been demonstrated that ethylene-induced hyponastic growth in *Rumex palustris* partly relies on auxin transport (Cox et al.,

2004). In addition to auxin, also brassinosteroids have been shown to be required for hyponastic leaf movement in response to elevated ethylene levels (Polko et al., in press). Brassinosteroids also control elongation responses to low R:FR (Kozuka et al., 2010) and low blue light conditions (Keuskamp et al., 2011) and may thus constitute another potential factor interacting with ethylene-dependent, low R:FR-induced petiole elongation and leaf hyponasty.

Although the delay in hyponasty is only a matter of several hours, this might already have implications for plants when competing with neighbours that do respond quicker, since the latter will have a higher chance of positioning their leaves above those of their neighbours. It has been demonstrated in Arabidopsis that relatively small differences in plant response to proximate neighbours can be of great ecological importance for plant fitness (Keuskamp et al., 2010). On the other hand, in dense stands, hyponasty will first be induced by touching leaf tips in an ethylene-independent manner and then be further stimulated by subsequent exposure to low R:FR conditions. Future studies should investigate whether touch-induced leaf angles need low R:FR-induced elevated ethylene levels to further increase their leaf angles in response to low R:FR.

In conclusion, ethylene signalling is only marginally involved in responding to major changes in R:FR conditions, but is likely of importance when plants need to respond rapidly and when responding to more incremental and smaller changes in R:FR conditions that represent canopy development. We conclude that ethylene is a modulator of plant responses to reduced R:FR conditions, thereby fine-tuning plant responses to proximate vegetation.

Acknowledgements

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CHAPTER

1 2 3 4 5 6

Canopy light cues affect emission of constitutive and methyl jasmonate-induced volatile organic compounds in *Arabidopsis thaliana*

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Abstract

When plants grow in dense stands they respond to various cues derived from competing neighbours. The best-described signals are light quality-related, such as a reduced Red : Far-Red light ratio (R:FR); these signals strongly affect plant morphology and resource allocation. Plants are also able to respond to chemical cues from neighbours, such as volatile organic compounds (VOCs). Changes in light conditions might interact with chemical cues. Here, we investigated how neighbour-related changes in the light environment change the emission of VOCs in *Arabidopsis thaliana*. Constitutive VOC emission was found to be reduced in plants that grow in dense stands, under a green filter or in low R:FR conditions, indicating that canopy light cues tend to suppress VOC emissions. It was shown previously that low R:FR conditions suppress plant chemical neighbours against herbivores and necrotrophic pathogens, probably by desensitizing plants to the plant hormone jasmonic acid (JA). Since the emission of many herbivore induced VOCs is JA-dependent, we investigated whether low R:FR conditions also suppress methyl-JA (MeJA) induced VOCs. Indeed, MeJA-induced emission of Green Leaf Volatiles and terpenoids was partially suppressed under low R:FR conditions. Moreover, the VOC-based preference of neonates of the specialist lepidopteran herbivore *Pieris brassicae* was significantly affected by the R:FR ratio. We argue that studies on plant-plant and plant-insect interactions through VOCs should take into account the light quality within dense stands when being extrapolated to natural and agricultural field conditions.

Introduction

Plants are bound to the location where they germinate and therefore need traits to adjust to environmental changes. Such acclimation requires both the ability to sense the environment and phenotypic plasticity to respond to these envi-

ronmental changes. When plants grow in dense stands, such as is the case in most agricultural and productive natural fields, they will have to deal with the close proximity of neighbouring plants that compete for resources. Aboveground, plants can sense neighbours through alterations in the light intensity and quality. Plants typically absorb red and blue light for photosynthesis, whereas far-red is reflected and transmitted through neighbouring leaves. Changes in the ratio between red and far-red light (R:FR ratio) are the first light cues associated with upcoming shading and plants can detect these changes using their phytochrome photoreceptors (reviewed by Franklin, 2008). The light spectrum of horizontally reflected light is already FR-enriched before true shading occurs, due to FR reflection by neighbouring plants and is sensed as an early neighbour detection signal (Ballaré et al, 1990). At the physiological level, plants respond to a lowering of the R:FR ratio with a broad range of responses, including enhanced stem and petiole elongation, apical dominance and early flowering which constitute the so-called shade avoidance syndrome (reviewed in Ballaré, 1999; Franklin, 2008; Keuskamp et al., 2011; Martinez-Garcia et al., 2010). These responses help plants to consolidate a favourable position in dense stands to support light capture and sustain their growth. In addition to light signals, also volatile organic compounds (VOCs) have been hypothesized to be involved in plant neighbour detection during competition (Kegge & Pierik, 2010), but this has been experimentally shown only for the volatile plant hormone ethylene (Pierik et al., 2003; 2004b). The production of ethylene is generally stimulated by low R:FR conditions (e.g. Finlayson et al., 1999; Pierik et al., 2004b; 2009; Foo et al., 2006; Kurepin et al, 2007). It remains to be demonstrated whether and how emissions of VOCs other than ethylene are affected by the light conditions in stands with high plant densities.

Competing neighbours are not the only organisms that can threaten a plant's potential for growth and reproduction in dense stands. Particularly at high plant densities, plants are at risk of being attacked by herbivorous insects and plants have evolved various mechanisms to defend themselves against herbivores. Studies on simultaneous exposure to competing neighbours and herbivore attack have led to the hypothesis of a trade-off between shade-induced growth responses and plant neighbours (e.g. Cippolini, 2004). This conflict between growth and neighbour has also been coined 'the dilemma of plants' (Ballaré, 2009; Herms & Mattson, 1992). Indeed, mechanistic studies focussing on shade avoidance and neighbour showed that low R:FR conditions lead to a severe suppression of inducible plant neighbours against herbivores (Izaguirre et al., 2006; Moreno et al., 2009), as well as against pathogens (Cerrudo et al., 2012; reviewed in Ballaré et al., (2012)). Neighbours against herbivore attack are induced upon insect feeding through increased production of jasmonic acid (JA) (Baldwin et al., 1997; Dicke et al., 1999). Accordingly, treatment of plants with exogenous JA induces various neighbour responses (Pieterse et al., 2009), and the JA receptor mutant *coi1-1* is more susceptible to herbivorous insects (Reymond et al., 2004; Bodenhausen &

Reymond, 2007; Van Oosten et al., 2008). JA is not only involved in direct neighbour against herbivores, but also plays a significant role in the production of herbivore-induced plant volatiles (HIPVs) in response to herbivore attack (Dicke et al., 1999; Snoeren et al., 2009).

Exogenous application of JA induces the emission of different volatile classes, such as green leaf volatiles (GLV), phenylpropanoids/ benzenoids and mono-, di and sesquiterpenes (Snoeren et al., 2009). Moreover, methyl jasmonate (MeJA), the methylated and volatile form of jasmonic acid, is effective in inducing direct neighbours in plant leaves (Farmer & Ryan, 1990; Miksch & Boland, 1996; Avdiusko et al., 1997) and inducing volatile emissions (Herde et al., 2008; Bruce et al., 2008; Faldt et al., 2003; Chen et al., 2003).
The emission of HIPVs is considered to serve as an indirect neighbour mechanism because HIPVs can attract natural enemies of herbivores (Dicke & Baldwin, 2010; Vet & Dicke, 1992). However, herbivores and plants can also exploit HIPVs: herbivores may avoid oviposition on plants that already contain eggs or feeding herbivores (Dicke, 2000) and plants grown near damaged neighbours may become more resistant to herbivory as was shown for tobacco grown near damaged sagebrush (Karban et al., 2003). JA-deficient mutants display reduced induction of many volatiles upon herbivore attack, especially terpenoids and green leaf volatiles (Snoeren et al., 2009; Thaler et al., 2002). Accordingly, JA-deficient tomato mutants receive more oviposition from herbivorous insects than did wild type plants (Sanchez-Hernandez et al., 2006), together indicating that JA mediates VOC emissions upon herbivore attack and these mediate interactions between plants and herbivores.

Because interplant distances in dense stands are small, the likelihood for VOCs to reach physiologically meaningful concentrations between plants is relatively high and this would favour eavesdropping on neighbouring plants to detect for example upcoming herbivore attack. However, the emission of many VOCs relies on JA signalling and the low R:FR conditions in a dense stand reduce induced plant neighbours that are JA-mediated. We therefore study in *Arabidopsis thaliana* if i) low R:FR, green shade (mimicking light conditions at early and late stages of canopy development) and dense vegetation impede constitutive VOC emissions, ii) low R:FR affects MeJA-induced VOC emissions and iii) low R:FR affects localisation of the food plant by naïve newly-hatched caterpillars (neonates) of *Pieris brassicae*, a specialist herbivore of brassicaceous plants such as *A. thaliana*. We demonstrate that the total amount of constitutively emitted VOCs is reduced under low R:FR conditions, green shade and in dense canopy conditions. For several individual compounds, similar results are found: emission is reduced when plants are exposed to shade. Furthermore, we show that the emission of MeJA-induced VOCs is reduced when plants are grown in low R:FR and that plant preference based on VOCs by *P. brassicae* is impeded. We conclude that light quality can have a strong impact on the emission of plant VOCs and their role in biotic interactions.

Materials and methods

Plant growth conditions & insect rearing

Arabidopsis thaliana accession Col-0 plants were grown on 1:2 potting soil:perlite mixture enriched with 0.14 mg of MgOCaO (17%; Vitasol BV, Stolwijk, The Netherlands) and 0.14 mg of slow-release fertilizer (Osmocote Plus Mini) per pot (70 ml) (Millenaar et al., 2009). After sowing, seeds were stratified for three days (dark, 4°C) and subsequently placed in a growth room (short days: 9 h light, 15 h dark, 200 µmol m-2 s-1 photosynthetically active radiation (PAR; Philips HPI-T Plus), 20 °C, 70% relative humidity) and kept underneath a glass plate to prevent seedling dehydration.

After three days, the glass plate was removed and pots were placed on automatically watered mats. Seedlings were transferred to individual pots (containing 70 ml of soil mixture) after 9-11 days. For volatile collection, plants were transferred to individual pots (containing 19 ml of soil:perlite mixture) that were covered with a Teflon plate with 1 mm holes to fit the hypocotyl through (fig. 1) and to separate the shoot compartment from the pot and soil. In high plant density experiments, plants were also grown in these 10 ml pots and these were positioned in square grids to reach a density of 2066 plants m-2.

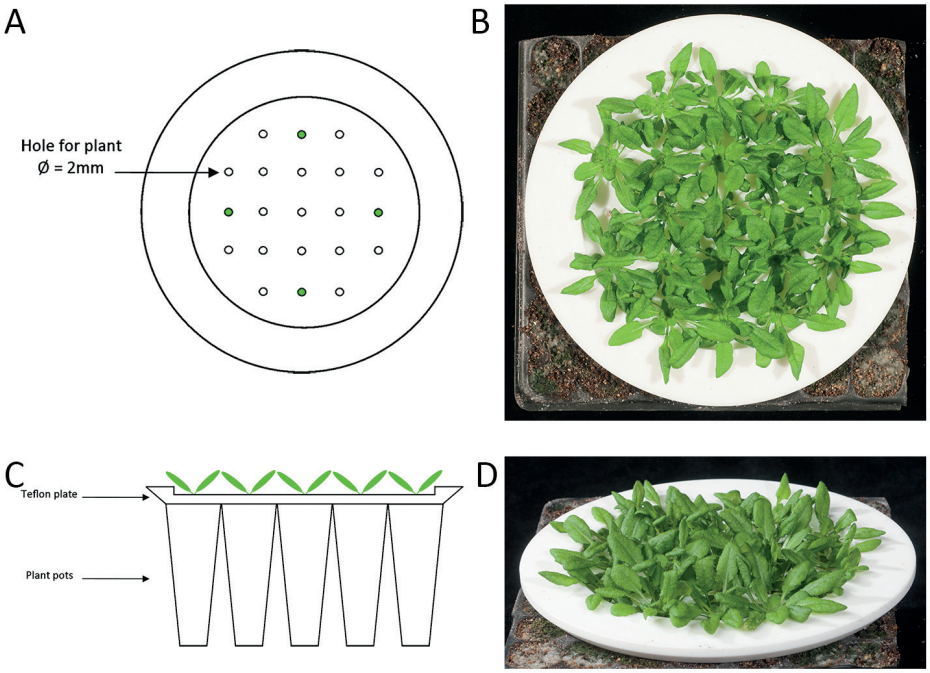

Figure 4.1 Graphic representation of teflon plate as used to hold plants for volatile collection. A) top view of plate (inner Ø 131 mm, outer Ø 183 mm), green spots indicate the holes that were used for individual plants. B) top view of plant canopy. C) Side view from plate with plants (plate thickness 3 mm), placed on top of the plant pots. D) side view of plant canopy.

The herbivorous large cabbage white *Pieris brassicae* was reared on Brussels sprouts plants (*Brassicae oleracea* var. gemmifera cv. Cyrus) in a growth chamber (16:8 L/D, 20 ®C and 70 % relative humidity) Eggs were laid on *B. oleracea* leaves, but removed from leaves approximately 18-24 h before hatching.

Experimental approaches

Light treatments were created in the following ways: plants were exposed to low red (λ = 660 nm) : far-red (λ = 730 nm) ratio (R:FR) in a growth chamber compartment with supplemental FR LEDs (730 nm, Philips Green Power) in a control white light background of 150 µmol m-2 s-1. This reduced the R:FR from 2.2 (control white light) to 0.2 (measured with Skye instruments, R:FR detector). Control plants were placed in a similar compartment with the same light intensity and the standard R:FR of 2.2 (660/730 nm) produced by the background white lamps. Green shade was achieved using green light absorbing filter (Lee 122 Fern Green, Lee Hampshire, United Kingdom), which reduced the PAR to 90 µmol m-2 s-1 and R:FR to 0.25.

The effects of methyl jasmonate were studied on plants that were grown in control light and in plants that had been exposed to low R:FR conditions for 6 d. Methyl jasmonate (pure quality, Van Meeuwen Chemicals BV, Weesp, The Netherlands) was sprayed onto the shoots at a concentration of 100 µM MeJA, and dissolved in 0.02% Silwet to facilitate an equal spread of the solution over the leaves. Mock-treated plants were sprayed with 0.02% Silwet. Per plant, ~1 ml solution was sprayed. Plants were placed in the fume hood to dry for one hour and then moved back into the growth chamber. MeJA was sprayed on plants 24 h prior to volatile collection, harvesting for gene expression or choice assays with caterpillars.

Dynamic headspace collection of plant volatiles

Plants were grown in the teflon plate–covered pots for 33-35 days with four (very low density, no plant-plant interactions through touch or light signals) or 21 (high density, strong plant-plant interactions) plants per plate. Plates had a diameter of 131 mm, with holes organized in square grids as shown in fig. 1a (inter-hole distance is 25 mm). The holes had a diameter of 2 mm, hole depth was 3 mm. Volatile samples were taken from aboveground plant parts; the teflon plate prevented belowground VOCs or volatiles from the soil to interfere (fig. 1b). Any unused holes in the Teflon plate were sealed with a few drops of 2% agarose gel. Air entering the cuvette (1.8 l) was first cleaned through an activated charcoal filter (4-8 mesh, Sigma Aldrich NL, Zwijndrecht) and a Tenax TA cartridge (250 mg Tenax). After 30 minutes of flushing, plant headspace collection occurred for 4 h at a flow rate of 4 L/h in a Tenax TA cartridge containing 250 mg Tenax-TA (20/35-mesh, Grace-Alltech, Deerfield, MI, USA). Volatile collection was performed at room temperature.

Analysis of plant volatiles

A Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, USA) coupled with Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, USA) quadrupole mass spectrometer (MS) was used for separation and identification of plant volatiles. Prior to releasing volatiles, the Tenax TA cartridges were dry-purged under a flow of nitrogen at 20 mL min-1 for 10 min at ambient temperature in order to remove moisture. The collected volatiles were thermally released from the Tenax TA on an Ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK) at 250 oC for 10 min under a helium flow of 20ml/min while re-collecting the volatiles in a thermally cooled universal solvent trap at 10 oC using Unity (Markes, Llantrisant, UK). Once the desorption process was completed, the cold trap was heated from 40 oC s-1 to 280 ° C and was held for 10 min while the volatiles were transferred to a ZB-5MSi analytical column (30 m x 0.25 mm I.D. x 1.00 µm F.T. (Phenomenex, Torrance, CA, USA)), in a splitless mode for separation. The analytical column was set at initial temperature of 40 °C and immediately raised at 5 oC min-1 to 280 °C and was held for 4 min under a column flow of 1 mL min-1 in a constant flow mode. The DSQ MS was operated in a scan mode with a mass range of 35–350 amu at 5.38 scans s-1 and ionization was performed in EI mode at 70 eV. MS transfer line and ion source were set at 275 and 250 oC, respectively. Identification of compounds was based on comparison of mass spectra with those in the NIST 2005, Wiley and Wageningen University Mass Spectral Database of Natural Products. Experimentally calculated linear retention indices (LRI) were used as additional criterion for confirming the identity of compounds. Relative quantification (peak areas of individual compounds) was performed using a single (target) ion in selected ion monitoring (SIM) mode. These individual peak areas of each compound were further used for characterization of the different treatment groups using statistical approaches.

Insect rearing and choice assays

The innate preference of neonate *Pieris brassicae* caterpillars was evaluated in two-choice experiments (Soler et al., 2012). Eggs of *P. brassicae*, laid on Brussels sprouts plants (*Brassica oleracea*) were removed approximately 18-24 h before hatching. This was done to obtain naïve neonates that had not fed on plant material prior to the experiment. Newly emerged neonates were individually placed on carton platforms (1 cm2), bridging control and treatment plants. Neonate caterpillars were released individually in the middle of the platform, and observed continuously until a choice was made (maximally 15 min). A choice was recorded when the larvae climbed onto a plant. To avoid the induction of plant neighbours and thereby possible influence on the choice of subsequently released conspecifics, larvae were removed from the leaf before they started feeding from the leaves. A minimum of eight plant pairs and eighteen larvae per pair were tested per combination of treatments. The following plant treatments were compared on 30-33-day-old plants: (a) control vs. low R:FR, (b) control vs. MeJA, (c) control

vs. low R:FR + MeJA, (d) low R:FR vs. low R:FR + MeJA, (e) MeJA vs. low R:FR + MeJA. Plants were placed in low R:FR conditions 6 d prior to the choice assays, while ~1 mL of 100 µM MeJA was sprayed per plant 24 h before choice assays.

RNA isolation and RT-qPCR

Leaf laminas of three fully-grown leaves were snap-frozen in liquid nitrogen and subsequently stored at -80 ˚C. RNA extraction, cDNA synthesis and RT-qPCR were performed as described in chapter 2. The Primer3Plus software (Untergasser et al., 2007b) was used to develop gene-specific primers and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression (Livak & Schmittgen, 2001) with *Ubiquitin5 (UBQ5)* and *Tubulin 6 (TUB6)* as internal standards.
Primer sequences are shown in table S4.1. Primers were tested for gene specificity by performing melt curve analysis on a Bio-rad MyIQ single-colour RT-qPCR detection system using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Expression of the following genes that are associated with VOC biosynthesis was tested: VSP2, vegetative storage protein 2, is known to code for a protein that slows herbivore feeding (Liu et al., 2005). *TPS4*, terpene synthase 4, is involved in the biosynthesis of geranyl linalool, a precursor of (*E*,*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT) (Herde et al., 2008). *TPS3*, another terpene synthase, is shown to be involved in the synthesis of β-ocimene and β-myrcene (Faldt et al., 2003). *CYP72A13* is postulated to be involved in the conversion of geranyl linalool to TMTT (Bruce et al., 2008) and *BSMT1* (S-adenosylmethionine-dependent methyltranfsferase) is involved in the methylation of salicylic acid (SA) and benzoic acid (Chen et al., 2003).

Statistical analysis

Headspace compositions were statistically analyzed through a multivariate analysis using SIMCA P+ ver. 12.0.1.0 (Umetrics, Umeå, Sweden). The quantitative results of the volatile blends of the different treatments were log transformed, mean-centred and scaled to unit variance prior to being analysed with a multivariate approach called Projection to Latent Structures-Discriminant Analysis (PLS-DA). PLS-DA with a classical PLS regression takes advantage of class information in maximizing the separation between groups of observations, where the dependent variable y is categorical and represents sample class membership (Szymańska et al., 2012). In PLS-DA, cross-validation is employed in order to determine the number of significant PLS components (Eriksson et al., 2006). Results of PLS-DA analysis can be displayed as a score plot, where pattern recognition of sample groups can be visualized. The score plot can be complemented by a loading plot, which can show which x-variables, in this case volatile compounds, are playing a role in making the group separation of samples in the score plot possible.

Data on peak area units was statistically analysed by using a one- or two-way ANOVA, followed by a Bonferroni post-hoc test to allow comparisons between

different treatments. For herbivore choice experiments, data were analysed with a binominal test. These comparisons were made using IBM SPSS statistics 20 software.

Results

Volatile emission by *Arabidopsis thaliana* decreases with increasing shade

To determine how proximate neighbours and their effects on light quality and quantity affected the emission of VOCs, individually grown plants were placed in three different light conditions; control light, low R:FR light (mimicking proximate, but not yet shading neighbours), green shade (mimicking shade imposed by neighbours). As a fourth treatment, plants were grown in a dense stand. Under these four conditions, VOCs were collected during 4 h and analysed by GC-MS. Volatiles were quantified relatively by calculating the average peak area per compound per treatment. When plants were exposed to light conditions that occur at different stages of canopy development (low R:FR early on, green shade in later stages) or to high density itself, volatile emission after four hours of sampling decreased. The total amount of emitted volatiles was reduced in all these shading treatments (fig. 4.2 A).

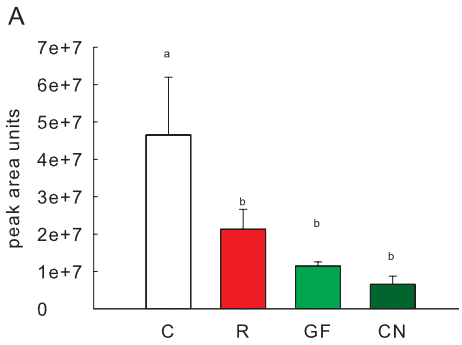

Figure 4.2 A) Total volatile amount (bars represent average of the sum of peak area units of all individual compounds). Volatiles were collected during 4 h headspace sampling from control plants (C), plants exposed to low R:FR (R), plants under green filter (GF) and plants that were growing in an actual canopy (CN). Significantly different classes are represented by letters (n=5, error bars represent standard error).

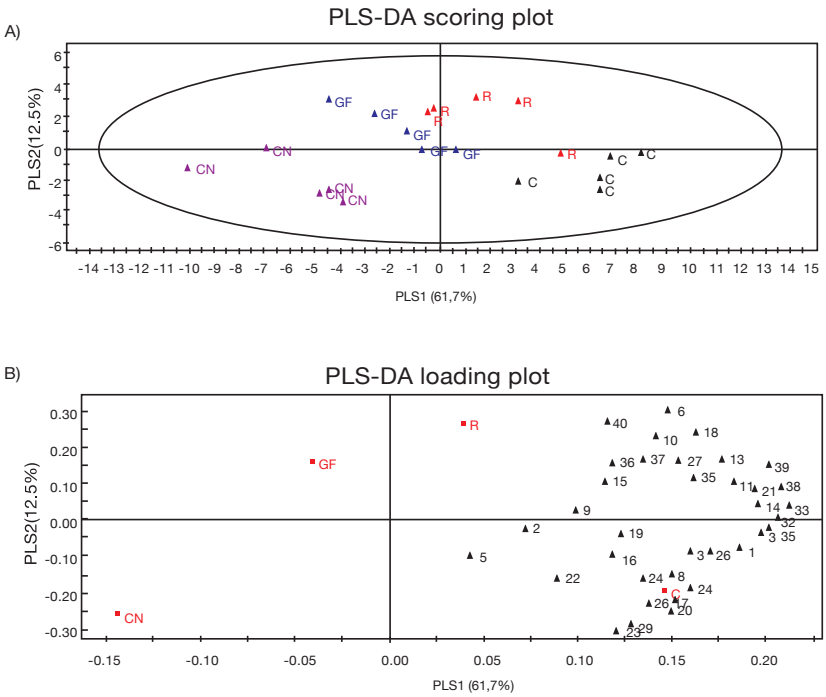

Figure 4.2 B) PLS-DA scoring plot of volatile collection, different treatments are classified by identical letters as in A). C) PLS-DA loading plot in which individual compounds are represented by different numbers. Compounds corresponding to different numbers are shown in table 2.

In order to distinguish groups between the different treatments, data were analysed with a projection to latent structure discriminant analysis (PLS-DA) (fig. 4.2 B). The first two principal components explained 61.7 and 12.5 % of the variance, respectively. The headspace composition of canopy-grown plants was most closely related to the headspace of plants exposed to green shade. Headspace composition of control plants was most closely related to the headspace of plants exposed to low R:FR conditions.

Table 4.1 Volatile compounds detected in headspace of *A. thaliana* plants, ranked by retention time. Volatile emission is ranked from high (left) to low (right) within each row, where significant differences ($p<0.05$, one-way ANOVA with Bonferroni posthoc test) are indicated by different letters. Volatiles were collected from control plants (Control, light blue), plants exposed to low R:FR (Low R, red), plants under green filter (Green filter, light green) and plants that were growing in an actual canopy (Canopy, dark green). (*E,E*)-TMTT: (*E,E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene, (*E,E*)-TMMHT: (*E,E*)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene

No	Compound	Ranking			
1	3-Methyl-2-butanone	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
2	1-Penten-3-ol	Control	Green filter	Canopy	Low R
3	2-Pentanone	Control[a]	Low R[ab]	Green filter[b]	Canopy[b]
4	1-Heptene	Control[a]	Low R[ab]	Green filter[b]	Canopy[b]
5	3-Pentanone	Control	Canopy	Low R	Green filter
6	Methyl thiocyanate	Green filter	Control	Low R	Canopy
7	2-Methyl-butanenitrile	Control[a]	Low R[ab]	Green filter[ab]	Canopy[b]
8	Methyl-cyclohexane	Control[a]	Green filter[b]	Low R[b]	Canopy[b]
9	3-Methylbutanenitrile	Control	Low R	Green filter	Canopy
10	Dimethyl disulfide	Control	Green filter	Low R	Canopy
11	1-Octene	Control	Low R	Green filter	Canopy
12	(R)-3-Methylcyclopentanone	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
13	Cyclohexanol	Control	Low R	Green filter	Canopy
14	1-Nonene	Control[a]	Low R[ab]	Green filter[b]	Canopy[b]
15	Cyclohexanone	Control	Low R	Green filter	Canopy
16	Anisole	Control	Green filter	Low R	Canopy
17	Cumene	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
18	2-Cyclohexen-1-one	Control	Low R	Green filter	Canopy
19	alpha-Pinene	Control[a]	Green filter[ab]	Canopy[b]	Low R[b]
20	Propylbenzene	Control[a]	Canopy[b]	Green filter[b]	Low R[b]
21	6-Methyl-5-hepten-2-one	Control[a]	Low R[ab]	Green filter[ab]	Canopy[b]
22	Limonene	Control	Green filter	Canopy	Low R
23	m-Cymene	Control[a]	Canopy[b]	Green filter[b]	Low R[b]
24	2-Isopropenyl-5-methylhex-4-enal	Control	Low R	Green filter	Canopy
25	1-Undecene	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
26	2-Butanoylfuran	Control[a]	Low R[ab]	Canopy[ab]	Green filter[b]
27	3-Acetyl-2,5-dimethylfuran	Control	Low R	Green filter	Canopy
28	Pulegone	Control[a]	Green filter[b]	Canopy[b]	Low R[b]
29	Isodurene	Control[a]	Canopy[b]	Green filter[b]	Low R[b]
30	4-Terpineol	Control	Low R	Canopy	Green filter
31	1-Dodecene	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
32	1-Tridecene	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
33	1-Tetradecene	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
34	Longifolene	Control[a]	Canopy[ab]	Low R[b]	Green filter[b]
35	Neoclovene	Control	Low R	Green filter	Canopy
36	(E,E)-alpha-Farnesene	Low R	Control	Green filter	Canopy
37	(E,E)-TMTT	Control	Green filter	Low R	Canopy
38	1-Hexadecene	Control[a]	Low R[b]	Green filter[b]	Canopy[b]
39	Pentadecanal	Control[a]	Low R[ab]	Green filter[b]	Canopy[b]
40	(E,E)-TMMHT	Control	Low R	Green filter	Canopy

In table 4.1, per volatile compound the different treatments are ranked based on the average peak area units (representing relative amount of emission by plants) in the respective treatments. In columns 3-6, the four different treatments are arranged from highest emission (left, column 3) to lowest emission (right, column 6). Considering the peak area units per compound, the peak area for control was in 12 out of 40 cases significantly higher than for the other three treatments, whereas the emission of these 12 compounds was not different among the three treatments.

In fig. 4.3, cumene and 1-dodecene illustrate the above-described pattern: both volatiles had higher emission in control treatment than in any of the other treatments, while no difference between the different shade treatments was observed. No compounds with higher emission in any of the light/density treatments than in control plants were detected (table 4.1). For 2-pentanone, 1-heptene, pentadecanal and 1-nonene, the peak area for control plants was higher than for plants exposed to green shade or for canopy plants, while no difference in emission was found between control and low R:FR-treated plants or between low R:FR and green shade/canopy plants (table 4.1); this pattern is illustrated for 1-nonene in fig. 4.3. Emission of α-pinene was reduced in the low R:FR and canopy treatments compared to control plants, while no difference was detected between control and green-shade treated plants (fig. 4.3).

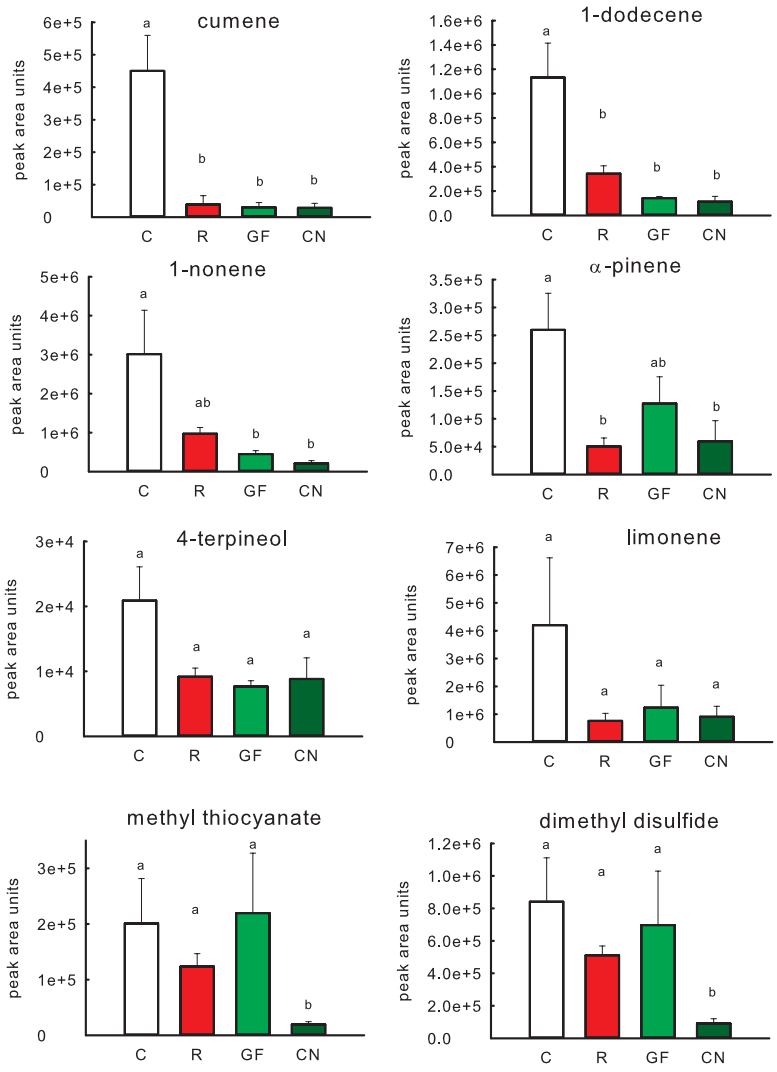

Figure 4.3 Emission of different VOCs by undamaged *Arabidopsis thaliana* plants, in terms of peak area units per gram plant dry weight detected in GC-MS analysis. Bars represent means ± SE (n=5). Volatiles were detected in 4 h headspace collection of undamaged *A. thaliana* plants. Volatiles were collected from control plants (C), plants exposed to low R:FR (R), plants under green filter (GF) and plants that were growing in an actual canopy (CN). Significant differences are represented by different letters ($p<0.05$, one-way ANOVA, Bonferroni post hoc).

In 23 out of 40 cases, average peak areas ranked as control > low R:FR > green shade > canopy (table 4.1), which is significantly higher than expected ($p<0.001$, chi square test). However, in 9 of these 23 cases no significant difference in peak area was observed between the treatments, illustrated for 4-terpineol and limonene in fig. 4.3. In chapter 3, it was demonstrated that ethylene emission is increased in plants in a canopy. Consistent with this observation the ethylene emission increased upon low R:FR conditions and under green shade (fig. 4.4).

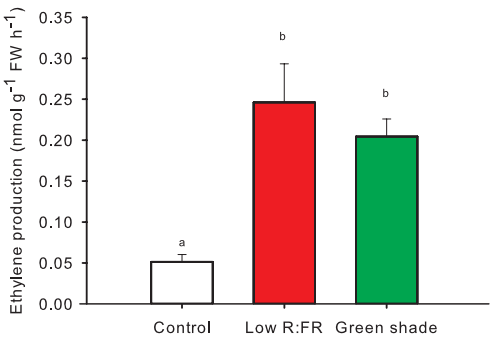

Figure 4.4 Ethylene emission of control, low R:FR and green filter treated plants after 4 h of treatment. Bars represent means ± SE (n=7) . Different letters indicate significant differences (one-way ANOVA, Bonferroni post hoc).

MeJA-induced volatile emission is suppressed under low R:FR

To test whether canopy and canopy signals also affect MeJA-induced VOCs, VOC emission in plants that received a MeJA treatment, low R:FR treatment or MeJA + low R:FR double treatment was measured. Although no interaction between MeJA and R:FR could be demonstrated (p=0.783), a significant main MeJA-effect was found (two-way ANOVA, p=0.001), whereas there was a trend for a main R:FR-effect (p=0.053) and, meaning that exposure to low R:FR had no significant impact on the effect of MeJA on the total amount of emitted volatiles (fig. 4.5). In table 4.2, per volatile compound the different treatments are ranked based on the average peak area units in the respective treatments. In columns 2-5, the four different treatments are arranged from highest emission (left, column 2) to lowest emission (right, column 5).

Figure 4.5 Bars represent average of the sum of peak areas of all individual compounds, corrected for plant dry weight. Volatiles were collected during 4 h from control plants (Control), plants exposed to low R:FR (R), plants sprayed with 100µM MeJA (MJ) and plants exposed to low R:FR and treated with 100 µM MeJA (R+MJ). Legend shows outcome of two-way ANOVA testing, * indicates a significant difference ($p<0.05$), + indicates a trend ($0.10>p>0.05$) and NS is not significant. Bars represent average (n=5) ± SE. Significant different classes are indicated by different letters (one-way ANOVA, Bonferroni post hoc).

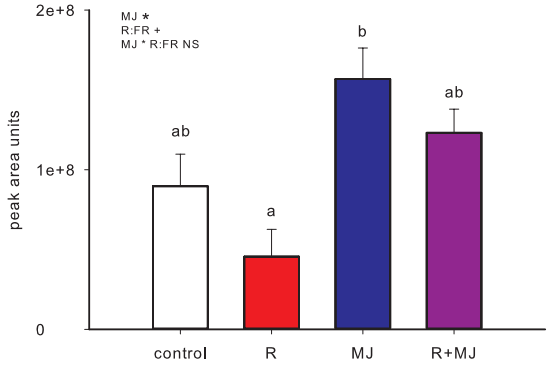

Table 4.2 Volatile compounds detected in 4 h headspace collection of *A. thaliana* plants of four different treatments, ranked by retention time. Volatile emission is ranked from high (left) to low (right) within each row, where significant differences ($p<0.05$, one-way ANOVA with Bonferroni post hoc test) are indicated by different letters. Volatiles were collected from control plants (Control, white), plants exposed to low R:FR (Low R, red), plants sprayed with 100µM MeJA (MeJA, blue) and plants exposed to low R:FR and treated with 100 µM MeJA (Low R + MeJA, purple). (*E,E*)-TMTT: (*E,E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene, (*E,E*)-TMMHT: (*E,E*)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene.

2-Butenal	Low R + MeJA	MeJA	Control	Low R
1-Penten-3-ol	Control	MeJA	Low R + MeJA	Low R
1-Methoxy-2-propanone	Control	MeJA	Low R	Low R + MeJA
3-Pentanone	MeJA	Control	Low R + MeJA	Low R
Unknown	MeJA	Control	Low R	Low R + MeJA
2-Methyl-butanenitrile	MeJA^{a}	Control^{ab}	Low R + MeJA^{b}	Low R^{b}
Methyl-cyclohexane	MeJA^{a}	Control^{ab}	Low R^{b}	Low R + MeJA^{b}
Butyl acetate	MeJA^{a}	Control^{ab}	Low R^{b}	Low R + MeJA^{b}
1-Nonene	MeJA	Control	Low R	Low R + MeJA
(E,E)-2,4-Hexadienal	Low R + MeJA	Control	MeJA	Low R
Anisole	MeJA^{a}	Low R + MeJA^{b}	Control^{b}	Low R^{b}
1S-alpha-Pinene	MeJA^{a}	Control^{ab}	Low R + MeJA^{ab}	Low R^{b}
Sabinene	MeJA^{a}	Low R + MeJA^{b}	Control^{b}	Low R^{b}
beta-Myrcene	MeJA^{a}	Low R + MeJA^{a}	Control^{b}	Low R^{b}
Unknown	MeJA	Control	Low R + MeJA	Low R
Limonene	MeJA	Control	Low R	Low R + MeJA
Phenylmethanol	Control	MeJA	Low R	Low R + MeJA
trans-beta-Ocimene	MeJA^{a}	Low R + MeJA^{a}	Control^{b}	Low R^{b}
p-Mentha-2,4(8)-diene	MeJA^{a}	Low R + MeJA^{a}	Control^{b}	Low R^{b}
Linalool	Low R + MeJA^{a}	MeJA^{a}	Control^{b}	Low R^{b}
3-Acetyl-2,5-dimethylfuran	MeJA	Control	Low R	Low R + MeJA
(Z)-Tagetone	MeJA^{a}	Control^{ab}	Low R^{b}	Low R + MeJA^{b}
Alloocimene	MeJA	Control	Low R + MeJA	Low R
p-Methyl-acetophenone	MeJA	Control	Low R	Low R + MeJA
1-Dodecene	MeJA	Control	Low R	Low R + MeJA
Methyl Salicylate	MeJA^{a}	Low R + MeJA^{ab}	Control^{b}	Low Rb
Indole	Low R + MeJA	MeJA	Control	Low R
2-Methylpropyl benzoate	MeJA^{a}	Control^{ab}	Low R^{ab}	Low R + MeJA^{b}
alpha-Copaene	MeJA	Control	Low R	Low R + MeJA
(E,E)-alpha-Farnesene	Low R + MeJA^{a}	MeJA^{ab}	Low R^{b}	Controlb
Valencene	Control	Low R	MeJA	Low R + MeJA
gamma-Cadinene	MeJA	Control	Low R + MeJA	Low R
(E,E)-TMTT	MeJA^{a}	Low R + MeJA^{ab}	Control^{b}	Low R^{b}
(E,E)-TMMHT	Low R + MeJA	MeJA	Control	Low R

For seven compounds, the emission in MeJA-treated plants under control light conditions was significantly higher than in MeJA-treated plants that were also exposed to low R:FR conditions. In control light, MeJA treatment significantly increased the emission of eight compounds, mainly monoterpenes (table 4.2, fig. 4.6).

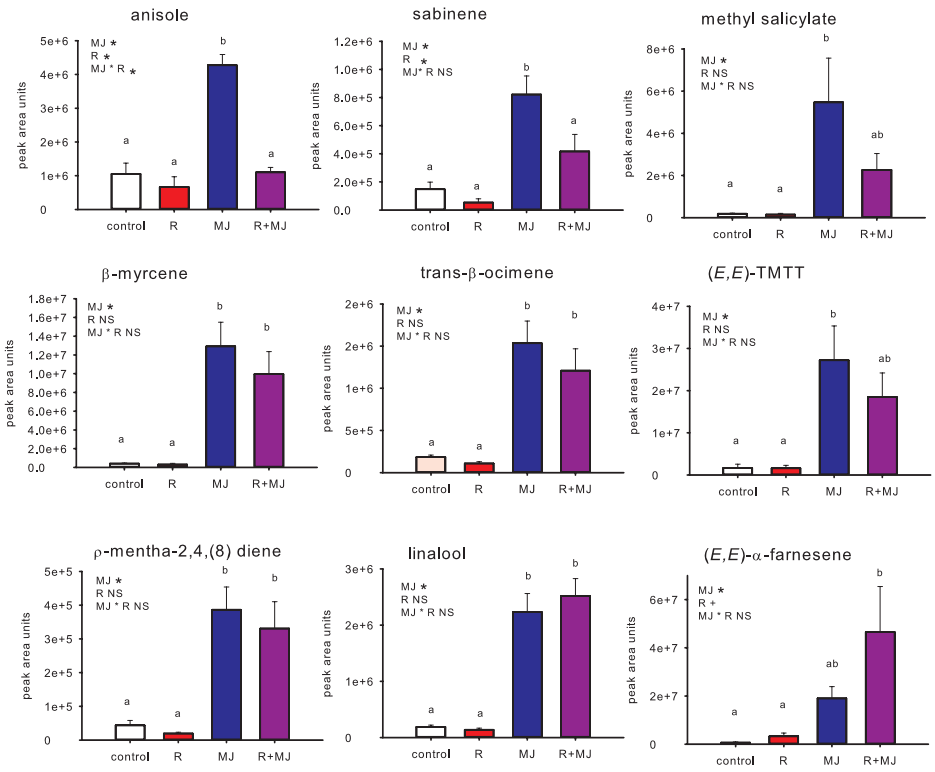

Figure 4.6 Peak area units of different VOCs, corrected for plant dry weight. Volatiles were collected during 4 h from control plants (Control), plants exposed to low R:FR (R), plants sprayed with 100µM MeJA (MJ) and plants exposed to low R:FR and treated with 100 µM MeJA (R+MJ). Legend shows main effects and interaction effect based on two-way ANOVA , * indicates a significant difference ($p<0.05$), + indicates a trend ($0.10>p>0.05$) and NS is not significant. Bars represent average (n=5) ± SE. Significantly different classes are indicated by different letters (one-way ANOVA, Bonferroni post hoc).

MeJA did not induce the emission of four of these compounds (anisole, sabinene, methyl salicylate and (*E,E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT)) when plants had been exposed to low R:FR conditions. (*E,E)*-α-farnesene was the only MeJA-induced compound under low R:FR that was not MeJA-induced in normal light conditions. The application of MeJA did not affect the emission of ethylene, irrespective of the light quality, whereas low R:FR did stimulate ethylene emissions (fig. 4.7).

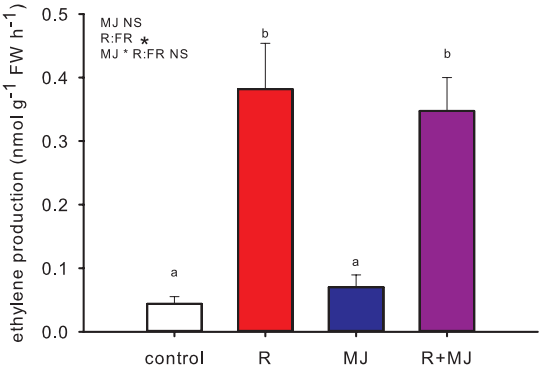

Figure 4.7 Low R:FR- and MeJA- induced emission of ethylene of the shoot of *A. thaliana* plants. Volatiles were collected from control plants (Control), plants exposed for 6 h to low R:FR (R), plants sprayed 24 h prior to ethylene measurements with 100μM methyl jasmonate (MJ) and plants exposed for 6 h to low R:FR and sprayed 24 h prior to ethylene measurements with 100 μM MJ (R+MJ). Data represent average of five independent replicates ± SE. Legend shows outcome of two-way ANOVA testing, * indicates a significant difference ($p<0.05$) and NS is not significant. Bars represent average (n=5) ± SE. Significant different classes are indicated by different letters (one-way ANOVA, bonferroni posthoc).

To establish whether differences in MeJA-induced volatile emission between control and low R:FR-grown plants are associated with differences in transcriptional regulation, we tested several genes that are involved in the biosynthesis of herbivore-induced plant volatiles (HIPVs). In addition, we investigated the expression of the MeJA-responsive gene coding for vegetative storage protein 2 (*VSP2*) to confirm its previously described down-regulation under low R:FR (Moreno et al., 2009) which is related to FR-mediated neighbour suppression. Indeed, *VSP2* expression was up-regulated after MeJA-treatment and this up-regulation was suppressed by exposure to low R:FR conditions (fig. 8). Low R:FR had a negative effect on the MeJA-induced transcript levels of terpene synthase 4 (*TPS4*) and S-adenosylmethionine-dependent methyltranfsferase (*BSMT1*) (fig. 4.8). Transcript levels of *TPS4* and *BSMT1* did not differ between control plants and plants grown under low R:FR conditions that were also MeJA-treated (fig. 4.8). MeJA-induced transcript levels of *CYP72A13* and *TPS3* were not reduced under low R:FR (fig. 4.8).

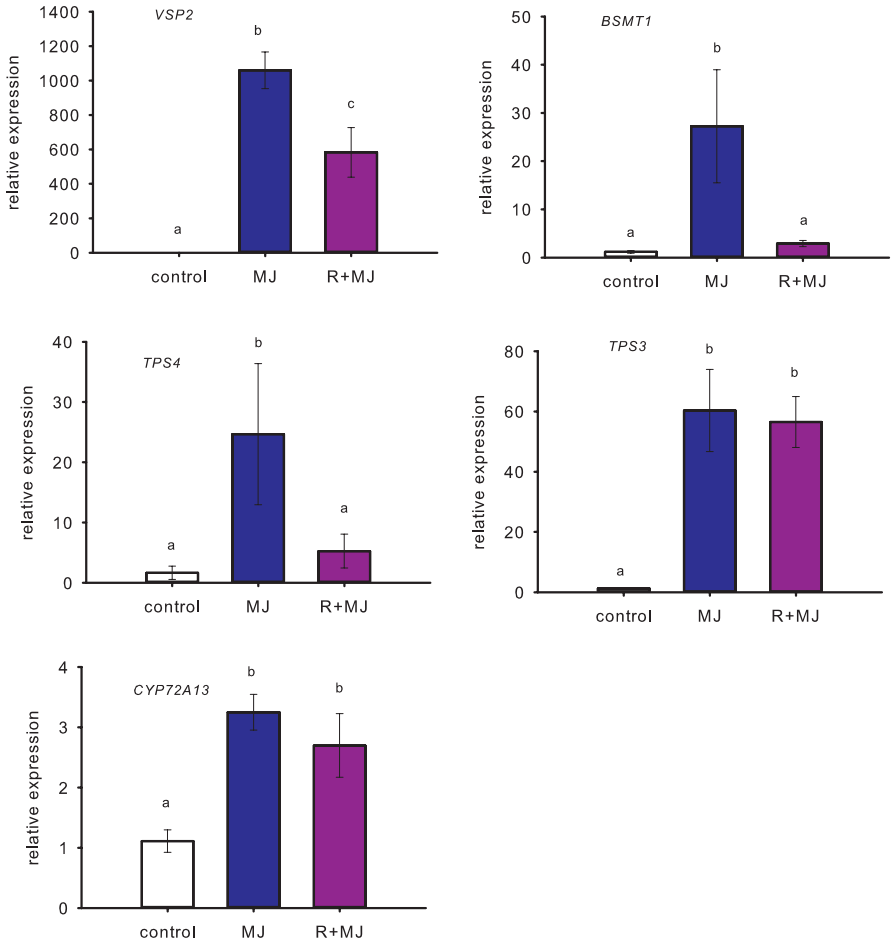

Figure 4.8 Relative expression of VSP2, BSMT1, TPS4, TPS3 and CYP72A13. Control plants were grown under normal light conditions(R:FR 2.0), MeJA plants were sprayed with 100 µM MeJA 24 h prior to harvesting for gene expression (MJ), double-treated plants were grown for six days at R:FR 0.2 and sprayed with 100 µM MeJA 24 h prior to harvesting for gene expression (R+MJ). (n=5). Data represent averages of five independent biological replicates ± SE. Different letters indicate significant differences (one-way ANOVA, bonferroni post-hoc test).

Neonate *P. brassicae* preference for MeJA-treated plants is abandoned in low R:FR

Since VOCs can serve as a cue for herbivores to locate their preferred host, we tested orientation behaviour of *Pieris brassicae* caterpillars towards low R:FR-grown and MeJA treated plants. When allowed to choose between control and low R:FR-grown plants, no preference of neonate *P. brassicae* caterpillars was observed (p=0.15) (fig. 4.9). Low R:FR-grown plants, sprayed with MeJA were preferred by 73 % of the caterpillars over plants sprayed with MeJA and grown in

normal light (p<0.001) (fig. 4.9). Although 59% of the neonates of this specialist herbivore preferred plants treated with MeJA over mock treated plants (p=0.012), this preference disappeared when plants were grown under low R:FR light: low R:FR plants treated with MeJA were not preferred above low R:FR without MeJA treatment (p=0.33) (fig. 4.9).

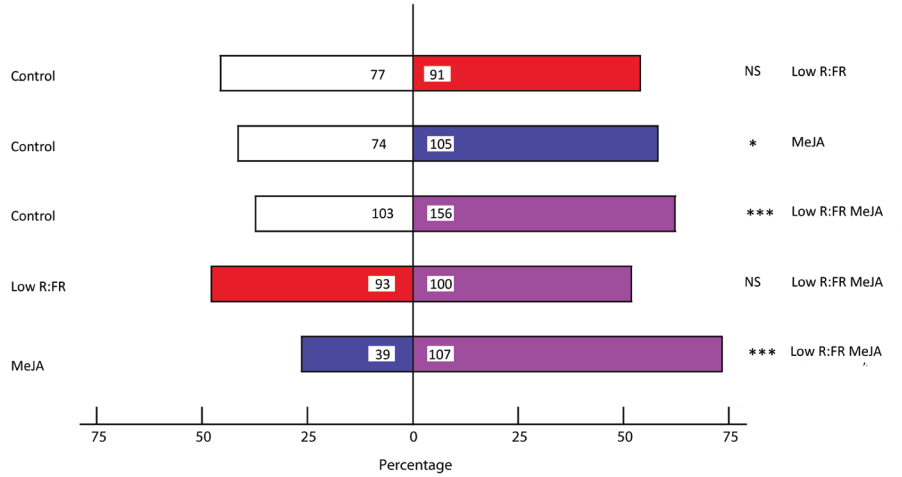

Figure 4.9 Preference of *P. brassicae* in two-choice tests. For each test, eight different plant pairs were used. Plants grown at R:FR 2.0 (Control), plants grown at R:FR 0.2 (Low R:FR), plants sprayed with 100 µM MeJA (MeJA) and plants grown at R:FR 0.2 and sprayed with 100 µM MeJA (Low R:FR MeJA) were tested. Bars represent percentage of herbivores that preferred the treatment. Numbers in bars represent numbers of herbivores that made the corresponding choice. Data were analysed with a binominal test. Significant differences are indicated by * (p<0.05) or *** (p<0.001). NS indicates that no significant differences were found for the corresponding choice assay.

Discussion

This study demonstrates that shade and shade-related plant cues from neighbouring plants reduce the emission of both constitutive and MeJA-induced volatile compounds in Arabidopsis. Moreover, preference of neonate *P. brassicae* caterpillars for MeJA-treated plants disappears when neonates have to choose between low R:FR grown plants and low R:FR grown plants that are sprayed with MeJA.

Because the root systems of plants at high density were kept separate, the effect of density on VOC emission will have been caused solely by aboveground factors. We, therefore, hypothesize that the severe reduction of VOC emission by growing in a high density follows from the combined effects of FR reflection by and transmission through leaves from neighbouring plants, and partial shading by neighbouring plants.

Reduced volatile emission in green shade or dense canopy conditions is not surprising, because VOC emissions are well-known to be controlled by light intensity: increasing light intensity leads to increased VOC emissions (e.g. Takabayashi

et al., 1994; Gouinguene & Turlings, 2002; Halitschke et al., 2000; Maeda et al., 2000). However, the reduction of VOC emissions in plants exposed to low R:FR without a reduction in light intensity is a novel finding and implies that phytochrome signalling affects VOC emission.

Since phytochrome signalling controls JA responsiveness (Cerrudo et al., 2012; Moreno et al., 2009) and many herbivory-induced volatiles are produced through the JA pathway (Halitschke et al., 2008; Boland et al., 1995; Dicke et al., 1999; Van Poecke & Dicke, 2002), it is possible that the observed effects of low R:FR on MeJA-induced VOC emissions occur through modulation of JA signalling. However, it remains elusive how low R:FR conditions down-regulate the emission of constitutive VOCs.

Most VOCs that are emitted upon MeJA-treatment were also found after application of JA (Van Poecke, 2002). Gene expression data for *BSMT1* and *TPS4*, involved in the biosynthesis of methyl salicylate and TMTT respectively, showed a clear suppression of the MeJA-induced up regulation when combined with low R:FR, which is consistent with the reduced emissions of MeJA-induced methyl salicylate and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). *CYP72A13* is involved in converting geranyllinalool to TMTT (Bruce et al., 2008), but the reduced MeJA-induced TMTT emission under low R:FR conditions are not accompanied by reduced *CYP72A13* transcript levels. It is possible that under low R:FR conditions, the reduction in TMTT emission is caused by a reduction in the production of geranyl linalool, which is considered to be the rate limiting step in TMTT emission (Herde et al., 2008). Another MeJA-inducible gene involved in VOC biosynthesis that was not affected by low R:FR conditions was *TPS3* (fig. 7), which encodes a terpene synthase involved in the synthesis of β-ocimene and β-myrcene (Van Poecke et al., 2001; Faldt et al., 2003). Indeed, also the MeJA-induced emission of trans-β-ocimene and β-myrcene was not affected by low R:FR (fig. 6). The observation that not for all MeJA-induced volatiles the emission was reduced under low R:FR might be caused by the occurrence of different signal transduction branches in the JA-mediated pathway and the role of ethylene in these branches.

Upon herbivory or wounding, a signal transduction branch is activated that is under control of the basic helix-loop-helix transcription factor MYC2, which negatively regulates a second branch of the JA pathway that involves Ethylene Response Factor1 (ERF1), an ethylene-responsive transcription factor (Verhage et al., 2011; Pieterse et al., 2012). The ERF1-branch of the JA pathway is mainly effective in inducing resistance against necrotrophic pathogens (Pre et al., 2008). It would be possible that the low R:FR-induced production of ethylene leads to enhanced induction of the ERF1-branch of the JA pathway, at the expense of the MYC2-branch. If the MYC2 branch would be most effective in inducing VOC emission, this could subsequently explain the observed down-regulation of emis-

sions of a number of MeJA-induced VOCs in low R:FR. However, it has been demonstrated that application of exogenous 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor of ethylene) can have a positive effect on the emission of particular JA-induced VOCs in lima bean (Horiuchi et al., 2001).

The increase in ethylene emission makes this compound a suitable candidate signal that could mediate neighbour-detection by Arabidopsis plants. Indeed, it has been shown that ethylene emissions are enhanced when Arabidopsis plants grow at high density (chapter 3), ethylene levels can accumulate inside dense tobacco stands to physiologically relevant levels and that the perception of ethylene is required for tobacco to successfully compete for light (Pierik et al., 2003, 2004b). It is possible that this involvement of ethylene in plant-plant signalling at high densities relies on the up-regulated emissions in response to neighbour-derived light signals, which is in sharp contrast to other VOCs of which the emissions are reduced in response to the presence of neighbours and neighbour-derived changes in light quality. Nevertheless, it would still be possible that VOCs act to detect neighbours prior to the occurrence of light signals, which in Arabidopsis stands is any developmental phase prior to the onset of touching leaf tips between neighbouring plants (chapter 2). In Arabidopsis canopies, there is only limited air volume within the shielded canopy where VOCs might be expected to accumulate. Thus, even though interplant distances in dense stands are very small, the reduced emissions make it unlikely that these components would serve as cues to detect neighbours. Interestingly, the emission of a number of volatile compounds was not drastically down-regulated, such as for (*E,E*)-α-farnesene and these might still signal between neighbours. This leads to an altered composition of the volatile blend that is emitted under low R:FR conditions. It has to be investigated whether the altered composition of the volatile blend has effect on proximate neighbours, since absence of specific (groups of) VOCs can lead to a stronger response to VOCs (Paschold et al., 2006). Besides changed VOC emissions in shading conditions, volatile signalling in dense stands is likely to happen regarding reduced plant distances.In addition to signalling between neighbouring plants, VOCs also affect members of higher trophic levels. Since animal responses to VOC are usually dependent upon the composition of the blend, rather than individual compounds (De Boer et al., 2004; Van Wijk et al., 2011), our observations can have consequences for interactions with animals such as herbivorous insects and their natural enemies. Possible effects of low R:FR conditions on the role of VOCs in attracting/repelling animals were studied here using the specialist herbivore *P. brassicae*. Although under control light conditions neonates of *P. brassicae* had a preference for MeJA-treated plants compared to control plants, this preference was lost when *P. brassicae* was allowed to choose between low R:FR-grown plants and low R:FR-grown plants treated with MeJA. The *P. brassicae* preference under control light conditions for MeJA-induced over mock-treated plants might be explained by the observation that MeJA-treated plants

emitted higher amounts of volatiles and thus the signal was stronger and possibly more attractive. However, this conflicts with behaviour of *P. brassicae* butterflies, since these butterflies laid significantly fewer egg batches on JA-treated than on untreated *Brassica oleracea* plants (Bruinsma et al., 2007). This might suggest that *P. brassicae* neonates are not necessarily attracted to the total amount of volatiles, but might rather be attracted by the composition of the volatile blend. Alternatively, neonate caterpillars might display a different plant preference than adult butterflies, as was recently suggested by Soler et al., (2012).

In conclusion, we found that shading has a negative effect on the amount of VOCs emitted by *A. thaliana*. Both constitutive volatile emission and MeJA-induced volatile emission are reduced by low R:FR. This reduction in emission is mostly consistent with differences in gene expression in the biosynthetic pathway of different volatile compounds and seems to have consequences for host selection by a specialist herbivore. Since under natural and agricultural conditions plants most frequently grow in conditions with reduced R:FR ratio's due to proximate neighbours, it is possible that VOC-mediated multitrophic interactions are different from those observed under growth chamber or greenhouse conditions focusing on individual plants. Both herbivores and predators or parasitoids of herbivores might be less capable in locating their hosts, because of the reduction in volatile emission and change in composition of the volatile blend (Dutton et al., 2000; Müller & Hilker, 2000) due to shading. We argue that these data emphasize the need for specific attention towards light quality in VOC research on plant-plant and plant-insect interactions. The data presented here indicate that plant performance at high densities may be dominated by phytochrome signalling of light quality, thereby affecting plant growth, plant-plant signalling and plant-insect interactions.

Acknowledgements

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Table S4.1 Primer sequences of primers used for RT-qPCR

Gene	Primer	Sequence
UBQ5	F	ACATCCAGAAGGAATCGACG
	R	CTTGATCTTCTTCGGCTTGG
TUB	F	ATAGCTCCCCGAGGTCTCTC
	R	TCCATCTCGTCCATTCCTTC
VSP2	F	ATGCCAAAGGACTTGCCCTA
	R	CGGGTCGGTCTTCTCTGTTC
BSMT1	F	TGGTCACTACTACGAAGAAGATG
	R	GAGCATTGGTTCACTAACAGC
TPS3	F	GCCACCATCCTCCGTCTC
	R	CCAAGCCACACCGATAATTCC
TPS4	F	TCGCAGCACACACCATTG
	R	GAGCAGCACGGAGTTCATC

CHAPTER

1 2 3 4 5 6

Exposure to low red : far-red light conditions affects emission of volatile organic compounds and their effects on biomass allocation in neighbouring barley (*Hordeum vulgare*) plants

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Abstract

Volatile organic compounds (VOCs) play various roles in plant-plant interactions. Constitutively produced VOCs might function as a cue to sense neighbours and plants can emit allelopathic VOCs that inhibit growth of neighbours or germination of seeds. In response to VOCs emitted by neighbouring plants, changes in biomass allocation have been described for VOC receiver plants. However, small interplant distances are required for volatile information transfer between plants. Emission of both constitutive and methyl jasmonic acid-induced VOCs is reduced in *A. thaliana* when plants are exposed to light conditions that reproduce the light environment of dense stands where plants compete for light. Here, the effect of a reduction in the red:far-red ratio (R:FR), which is an early cue of proximate vegetation, is investigated in two barley (*Hordeum vulgare*) cultivars. Total VOC emission by the barley cultivar Alva is reduced under low R:FR conditions compared to control light conditions, although this pattern varies between different VOCs. The emission of two compounds, linalool oxide and ethylene, are even increased. Interestingly, the reduced emission of VOCs of low R:FR exposed Alva had a strong impact on carbon allocation in receiver plants of a second cultivar, Kara. We conclude that R:FR light conditions influence VOC-mediated plant-plant interactions.

Introduction

Plants adjust to environmental changes using a variety of cues, including chemical cues, derived from proximate vegetation. Aboveground information transfer through neighbour-induced changes in light quality and the responses initiated by these cues are well described (reviewed in Franklin, 2008; Ballaré, 2009).

The dominant aboveground light signal appears to be a reduced red:far-red light ratio (R:FR), caused by reflection of far-red and absorption of red light by leaves of neighbouring vegetation. In addition to changes in light quality, also volatile organic compounds (VOCs) emitted by neighbouring plants might serve as cues for surrounding plants (reviewed in Kegge & Pierik (2010); Chapter 1). VOCs are particularly well studied in the context of plant-herbivore interactions. Upon herbivore-induced plant damage, the emission of many VOCs increases (Dicke, 1994; Dicke et al., 1999; Turlings & Tumlinson, 1992). These herbivore-induced VOCs (HIPVs) serve in various systems as attractant for predators and parasitoids of the herbivores (Dicke et al., 1999; Thaler, 1999; Turlings & Tumlinson, 1992). Interestingly, HIPVs have also been shown to induce resistance in proximate neighbours, indicating that VOCs can serve as chemical cues between plants. For example, in cabbage (*Brassica oleracea*) exposure to VOCs from herbivore-infested conspecifics primes direct and indirect defence responses in intact plants (Peng et al., 2011). Volatile information transfer is not restricted to intra-specific interactions. For example, wild tobacco (*Nicotiana attenuata*) can induce resistance upon perceiving VOCs produced by clipped sagebrush (*Artemisia tridentate*) (Karban et al., 2003).

Next to plant-plant interactions being mediated by herbivore-induced VOCs, there is also evidence for information transfer through VOCs between non-attacked plants. As an example, *Cuscuta pentagona*, a parasitic plant, locates its host tomato (*Lycopersicon esculentum*) using VOCs and can discriminate the host VOC blend from non-host VOCs (Runyon et al., 2006). Constitutively emitted VOCs can also have inhibitory effects on plant growth. When germination or development is hampered by chemical compounds that are released from neighbouring plants, these compounds are considered allelopathic (Inderjit et al., 2011). For example, VOCs from *Artemisa frigida* can inhibit seed germination and decrease seedling growth of four different grass species (Zhang et al., 2012). Sasa (*Sasa cernua*) can inhibit the growth of barley seedlings (Li et al., 1992) and Nishida et al. (2005) showed that monoterpenoids produced by *Salvia leucophylla* can inhibit root growth in *Brassica campestris*. Finally, the volatile plant hormone ethylene can accumulate in the atmosphere inside dense tobacco stands to levels that affect plant growth (Pierik et al., 2003, 2004b).

Within-species, interplant information transfer can affect growth of different cultivars that are used in agricultural production systems: in barley (*Hordeum vulgare*), cultivars with differences in resistance genes are used in mixed production systems to suppress powdery mildew (*Erisyphe graminis*) infestation (Wiik, 1987; Brown & Jorgensen, 1991), reduce aphid host acceptance (Ninkovic et al., 2002; Ninkovic & Ahman, 2009) and increase attractiveness to ladybirds, which are natural enemies of aphids (Ninkovic et al., 2011). Information transfer through VOCs between two of these cultivars led to an altered carbon allocation between

root and shoot (Ninkovic, 2003). Plants from the cultivar Kara exposed to VOCs from the Alva cultivar invest relatively more biomass into their roots compared to unexposed control plants.

Despite the altered root-shoot allocation, total dry weight and relative growth rate (RGR) were not affected even though Kara exposed to Alva VOCs had a higher specific leaf area (leaf area per leaf dry weight, m2 kg-1) (Ninkovic, 2003), which is associated with increased RGR (reviewed in Poorter et al., 2012).

It is assumed that effects of Alva on Kara plants could also occur in true production system, since plant-plant distances in crop fields are typically small due to the high crop densities, thus enhancing the likelihood of VOCs reaching biologically meaningful concentrations at receiver plants. Indeed, information transfer between plants through VOCs decreases with increasing distance (Karban et al., 2003), probably through dilution of the signal in the air with increasing distance. However, in chapter 4 it was demonstrated that shading and low red:far-red light (R:FR) conditions reduce the quantity and alter the composition of the VOC blend emitted by *Arabidopsis thaliana*. Furthermore, it was shown that these changes are biologically meaningful since they changed the behaviour of *Pieris brassicae*, a specialist herbivore feeding on *A. thaliana*. However, it was not studied whether low R:FR exposure of emitter plants affected VOC-dependent information transfer of vegetative, non-attacked Arabidopsis plants. However, the two barley cultivars mentioned above constitute an elegant biological system to ask the question if light quality affects plant-plant interactions through VOCs. Therefore, it is studied here whether i) low R:FR exposure affects VOC emissions of barley and ii) whether low R:FR induced changes in volatile emission would influence the effects of VOCs emitted by the cultivar Alva on biomass allocation in Kara neighbouring plants that are exposed to these VOCs. We demonstrate that low R:FR exposure leads to a reduction in the total emission of Alva VOCs, consistent with what was reported in chapter 4 for *A. thaliana*. Furthermore, Kara exposed to VOCs of control light grown Alva has a different carbon allocation than Kara exposed to VOCs from Alva grown at low R:FR conditions. We conclude that low R:FR strongly affects VOC-mediated interactions between Alva and Kara plants through its impact on VOC emissions.

Material and methods

Plant growth

Two spring barley (*Hordeum vulgare L.*) cultivars (Kara and Alva) were used for these experiments (Ninkovic, 2003; Petterson et al., 1999). Plants were grown in a climate chamber at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden using a 16 h day/ 8 h night regime. Light intensity was set at 150 µmol m-2 s-1 (Hortilux Schréder, HPS 400 Watt, Holland), with an R:FR

ratio of 1.9, measured with a Skye SK110 660/730 (red/far-red) Sensor (Skye Instruments Ltd, Powys, United Kingdom), temperature was 20 °C ± 2 °, relative humidity was 60 % ± 5 %. Seeds were germinated on sand and transferred to perforated cylindrical polyethylene tubes (1 m with a diameter of 0.05 m) filled with washed silver sand 55 (Sibelco Nordic AB, Västerås, Sweden). Plants were watered automatically with a nutrient solution (130 mg N, 20 mg P, 103 mg K, 9 mg S, 6 mg Ca, 6 mg Mg, plus micro nutrients, 1 mg l-1 water) (Wallco plant nutrient 51-10-43+mikro, Cederroth International AB, Falun, Sweden) every 2 h and excess solution was drained from the tubes.

Plant-plant signalling experiments

Plants of 8 d old were placed in transparent twin-chamber cages as described by Ninkovic (2003) for 10 d. In this cage set-up receiver Kara plants were exposed to air from empty cages, air containing VOCs of Alva grown under control light conditions or air containing VOCs of Alva grown at low R:FR (R:FR = 0.2). Alva plants under low R:FR conditions received, in addition to the standard white light, FR light from FR-emitting LEDs (730 nm, Philips Green power, Philips, Eindhoven, The Netherlands). The two chambers (each 10 x 10 x 80 cm) of the clear perspex twin-chamber cages were separated by clear perspex tubes with a diameter of 7 cm and 30 cm long and an aluminium foil curtain. Separation of the chambers allowed exposure of plants in the emitter cage to different light conditions than the receiver plants and prevented light signalling between emitter and receiver plants. After 10 d of treatment, both Alva and Kara plants were harvested and the following measurements were taken: leaf area, leaf dry weight, stem length, stem dry weight, root dry weight and total root length. Leaf area was measured with a Li-3100 Area Meter (LI-COR), plant lengths were measured with a ruler. Plant material was dried in a stove at 70° C for 3 days and subsequently weighted on a balance (Sartorius, Sartorius Megatronics, Nieuwegein, The Netherlands). Roots were scanned for root length using a scanner (EPSON Perfection 4900 3.4, Regent Instruments, Quebec, Canada) and root length was quantified using WinRHIZO Pro V 2007 software (Regent Instruments, Quebec, Canada).

Collection and analysis of barley volatiles

Volatiles from barley plants were collected by air entrainment (Glinwood et al. 2011). 10-11 barley plants (cv Alva) were grown per pot. After 10 days, eight pots per treatment were placed under low R:FR (R:FR 0.2) and under control light (R:FR 1.9) for five days. After five days of low R:FR pretreatment, plants were placed for 72 h in polyester bags (Melitta Scandinavia AB, Toppits 60 x 55 cm), which had been baked previously for at least 2 h at 140 ^{0}C to purge volatile components from the polyester. Pots were wrapped in aluminum foil to minimize VOC release from the pots. Control light and low R:FR conditions were identical to those described in the previous paragraph. Bags were closed from the side with a plastic tie and plants were placed back in low R:FR or control conditions.

Charcoal-filtered air was pumped into the bag (push flow 600 ml min-1) from the lower part of the bag through a teflon tube fastened with a plastic tie. VOCs were trapped in glass tubes containing Porapak Q (PPQ, 50 mg PPQ per tube, mesh 50/80, Supelco, Bellefonte PA, USA) from the outgoing air (pull flow 450 ml min-1). The PPQ-filled tubes were fastened with a plastic tie at the top of the bag without touching the leaves but were not airtight. There was thus a slight positive pressure in the bags that prevented unfiltered air from entering. Teflon tubes and tinfoil were baked overnight at 180 ^{0}C. Charcoal filters were baked overnight in 180 ^{0}C with a constant nitrogen flow. PPQ tubes were rinsed with redistilled dichloromethane (DCM) and baked overnight under nitrogen flow (400 ml min-1) at a temperature of 150 ^{0}C and cooled down to room temperature just before volatile collection started.

After 72 h of volatile collection, the collected volatile compounds were rinsed from each of the PPQ traps with 750 µl redistilled DCM into a 2 ml glass vial. Next, 1 µl of (30 ng/ µl) 1-nonene was added as internal standard and the samples were reduced to 50 µl volume under a gentle flow of nitrogen. Compounds were identified using coupled gas chromatography-mass spectrometry (GC-MS).

Two µl of each sample was injected onto a HP1 column (30 m x 0.25 mm i.d., J & W scientific) housed in a 7890A GC (Agilent Technologies, Santa Clara, CA, U.S.A.) coupled to an Agilent 5975C mass spectrometer. Ionisation was achieved by electron impact at 70 eV. The oven temperature was maintained at 30°C for 1 min, and then programmed at 5°C/min to 150°C and held for 0.1 min, then 10°C/min to 250°C. The carrier gas was helium. Identifications were made by comparison of spectra with those of authentic samples in a database (NIST 2005) and confirmed by comparing retention times with those of authentic standards. Quantifications were made by dividing the total ion count for each target peak by that of the internal standard and multiplying by the known amount of standard in each sample.

Ethylene gassing and sampling

For experiments to determine ethylene production and the barley growth response to prolonged ethylene exposure, seeds were stratified for three days at 4 °C to synchronize germination and subsequently germinated and grown in growth chambers at Utrecht University, the Netherlands. Three days after germination, plants were placed in separate pots (9*9*9.5 cm), filled with primasta soil® (Primasta BV, Asten, The Netherlands) and 100 ml nutrient solution (Millenaar et al., 2005) was added per plant. Plants were placed in growth chambers and automatically watered daily with tap water.

Eight day old Kara plants were exposed to different concentrations of ethylene in glass cuvettes (18 l, 30x30x21 cm). Pure ethylene (Hoek Loos BV, Schiedam, The Netherlands) was mixed with air, using flow meters and controllers (Brooks

Instruments BV, Ede, The Netherlands) to reach the desired concentrations of 0, 25 and 250 ppb. Temperature was 20 °C ± 1 °, relative humidity was set at 70 % ± 3 % at a flow rate of 75 l h-1. Ethylene concentrations were calibrated prior to the start of the experiment, using a GC955 gas chromatograph with Photo ionisation detector and 160 cm Haye Sep R column, filled with Haye Sep 80/100 mesh (Synspec, Groningen, The Netherlands).
Ethylene production was measured on freshly harvested shoot tissue (~ 1.0-1.2 g fresh weight, n=5) after 20 min of headspace accumulation in a syringe, as described previously (Millenaar et al., 2005), using the GC955 mentioned above.

Statistics
Data were analysed through ANOVA or Student's t test in the IBM SPSS statistics 20 software.

Results

Exposure to low R:FR reduces the emission of barley volatiles
Barley VOCs from the cultivar Alva were collected from plants grown under control light conditions and from Alva plants exposed to low R:FR and analyzed by GC-MS. In total, 20 different compounds were detected. Control plants were found to emit higher quantities of VOCs than low R:FR-grown plants (fig. 5.1). The emission of 6 out of these 20 VOCs was significantly lower for low R:FR exposed plants than for control plants, whereas there was a trend towards such suppression for 1-octen-3-ol (p=0.0501). The emission of the sesquiterpenes (*E*)-β-caryophyllene, α-humulene and caryophyllene oxide were significantly reduced by low R:FR (fig. 5.2).

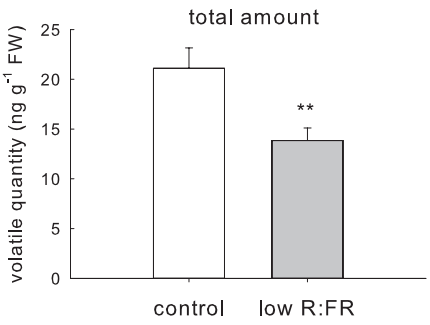


Figure 5.1 Total amount of emitted volatiles of Alva plants under control light (control) and low R:FR light conditions (low R:FR). Bars represent means ± SE (n=8). Significant difference (P=0.009, Student's t-test) is indicated by **.

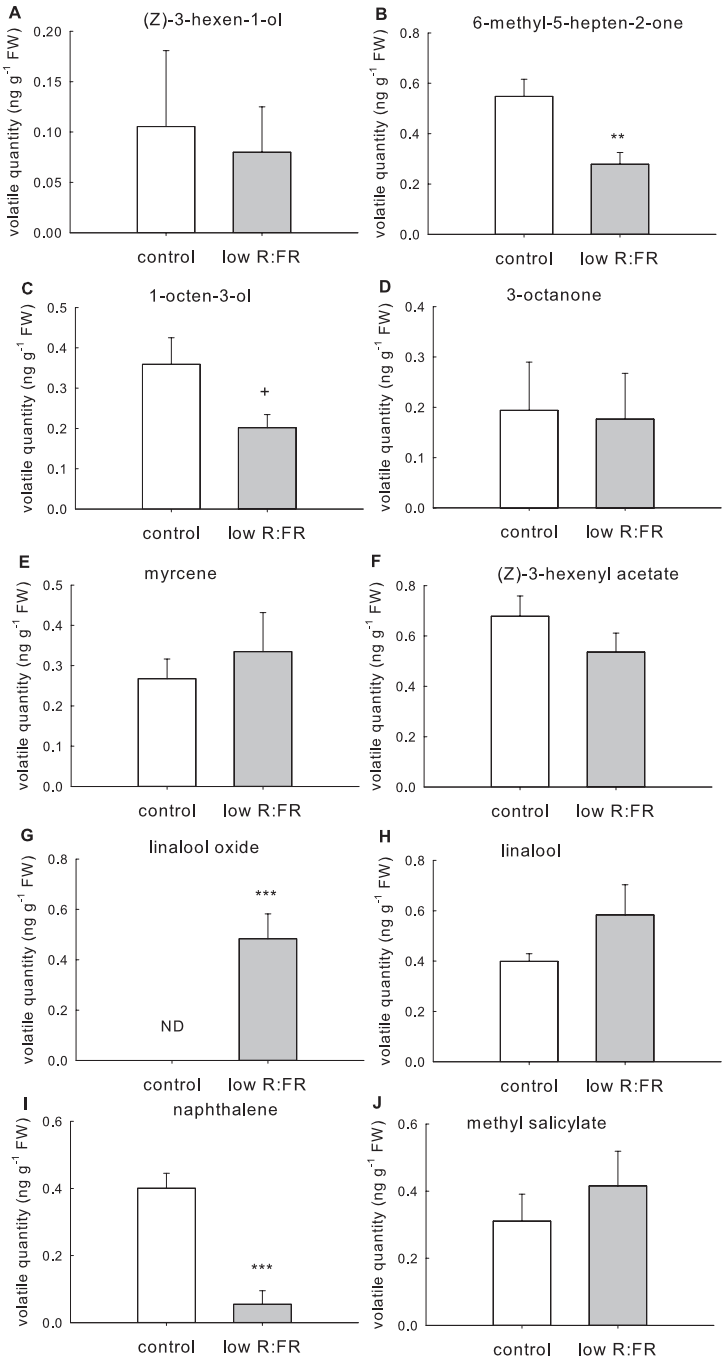
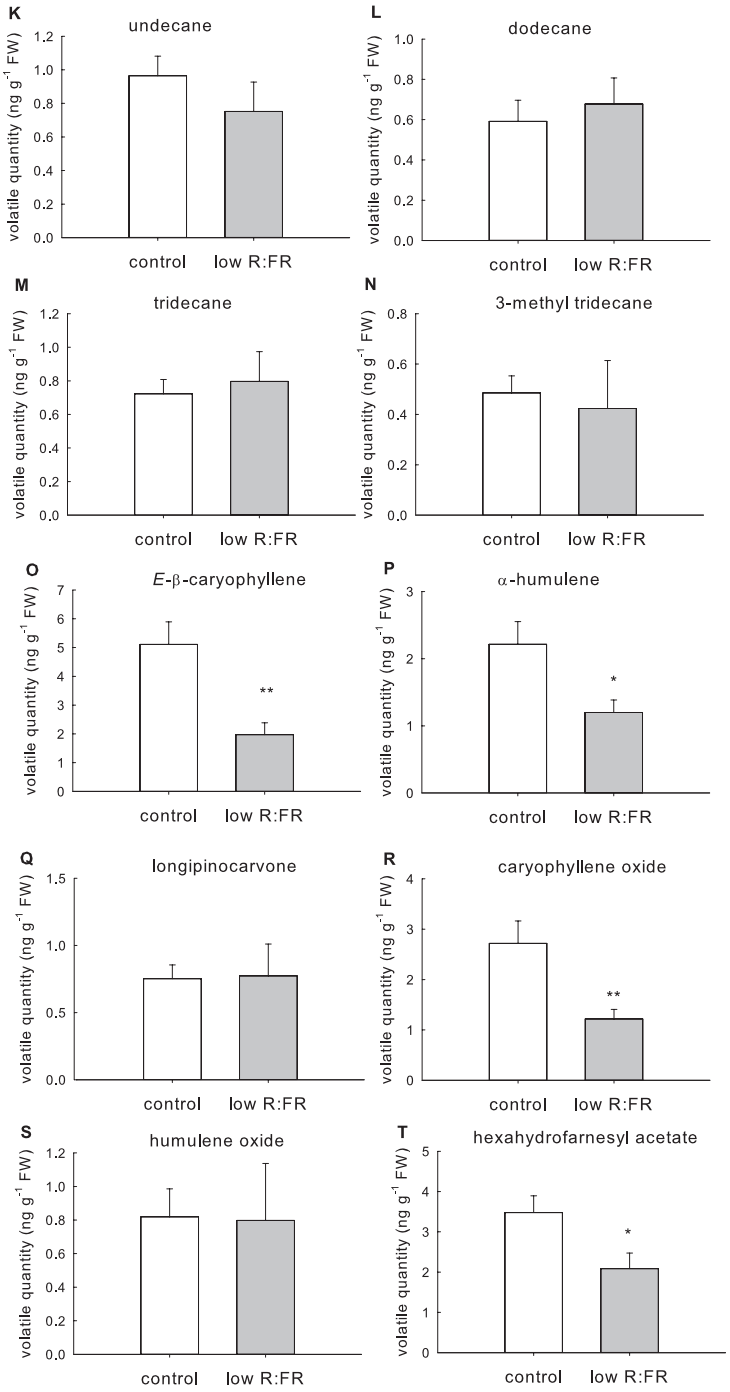


Figure 5.2 Emission of volatiles of Alva plants under control light (control) and low R:FR light conditions (low R:FR. Bars represent means ± SE (n=8). Significant differences are indicated by asterisks (Student's t-test, $p<0.05$ *, $p<0.01$ ** and $p<0.001$ ***), trends ($0.05<p<0.10$) are indicated by an +.

Figure 5.2 continued



Three other compounds, 6-methyl-5-hepten-2-one, the polycyclic aromatic hydrocarbon naphthalene and the terpene fatty acid hexahydrofarnesyl acetate showed also reduced emission under low R:FR (fig. 5.2). However, the emission of most monoterpenes was not significantly affected and low R:FR exposure did even stimulate linalool oxide emissions (fig. 5.2). Likewise, also the emission of the volatile plant hormone ethylene was enhanced under low R:FR (fig. 5.3), both after 6 h and after 10 d of low R:FR exposure.

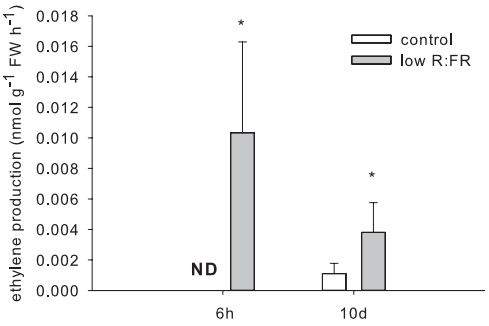


Figure 5.3 Ethylene production of the barley cultivar Alva after 6h and 10d of low R:FR exposure. Bars represent means ± SE (n=5). Significant differences per time-point ($p<0.05$, Student's t-test) are indicated by an asterisk, ND means Not Detected.

Low R:FR affects resource allocation in Alva plants

Alva emitter plants grown under low R:FR had a similar total dry weight as plants grown in control light (fig. 5.4 A). However, low R:FR-exposed Alva invested more dry mass in the shoot relative to the root than control plants, as expressed by an increased shoot/root ratio (fig. 5.4 B). Furthermore, low R:FR-grown Alva plants had a higher leaf mass fraction (LMF, g leaf g-1 total plant) and specific leaf area (SLA, m2 leaf g-1 leaf) (fig. 5.4 C & D), which corresponded with higher leaf dry weight and leaf area in low R:FR-grown Alva plants compared to plants grown in control light (fig. 5.4 E & F). A classic shade avoidance response to low R:FR is enhanced stem elongation and indeed, low R:FR-exposed Alva plants had a higher total plant height than control plants from the fifth day of low R:FR exposure onwards (fig. 5.5).

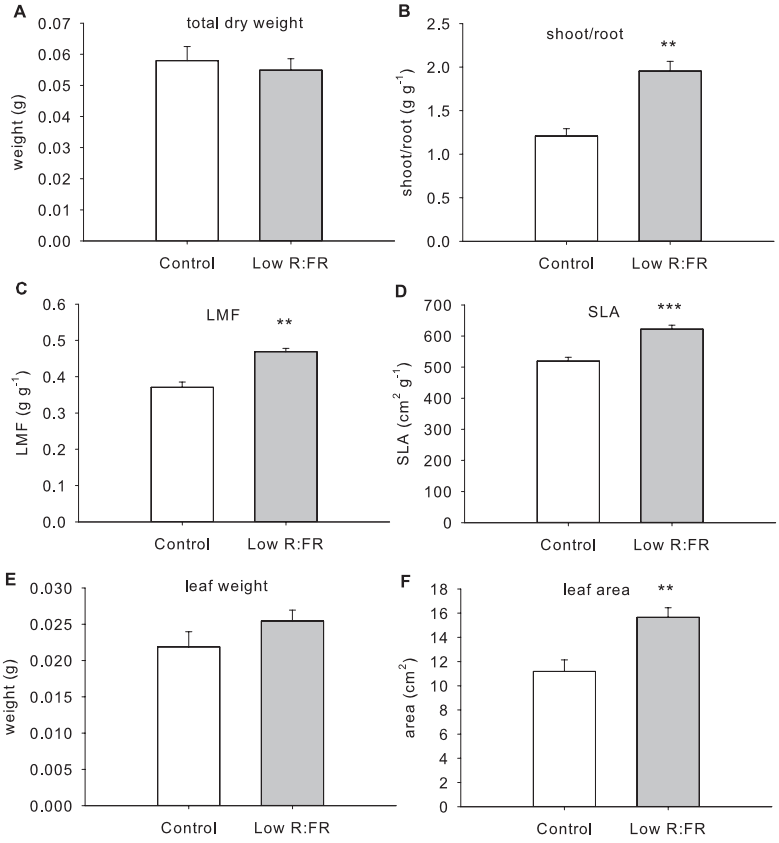


Figure 5.4 Growth response of Alva plants after 10 day exposure to low R:FR conditions (low R:FR) or under control light conditions (control). In panel C, leaf mass fraction (LMF, g leaf g-1 plant) is shown. Panel d represents specific leaf area (cm2 leaf g-1 leaf) Bars represent means ± SE (n=18-19). Data were analysed with a Student's t-test. Significant differences are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

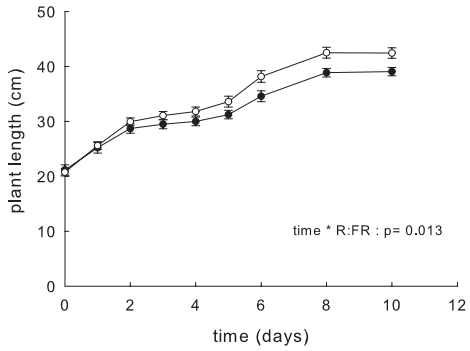


Figure 5.5 Length of Alva plants grown in control light (filled symbols) or low R:FR light conditions (open symbols). Data points are means ± SE (n=10). A significant interaction between time and light treatment was found (Wilk's Lambda p=0.013).

Exposure to low R:FR changes effects of Alva VOCs on Kara receiver plants

It has been previously demonstrated that exposure to Alva VOCs leads to altered resource allocation in Kara receiver plants (Ninkovic, 2003). Here, we studied whether effects of Alva plants on Kara plants through Alva-produced VOCs, would be affected by exposure of Alva emitters to reduced R:FR. Kara plants exposed to volatiles from Alva plants that were grown in control light (KAc) accumulated significantly less biomass than Kara plants exposed to volatiles from low R:FR-grown Alva plants (KAfr) (fig. 5.6 A). Kara plants that were not exposed to neighbour-derived volatiles were not significantly different from either KAc or KAfr in terms of aboveground dry weight measures. Leaf weight, leaf area and stem weight were higher for KAfr than for KAc (fig. 5.6 B-D). For stem length, no differences between different treatments have been observed (fig. 5.6 E). The total shoot weight was also higher in KAfr than KAc (fig. 5.6 F), which is in line with the observations on leaf weight and stem weight (fig. 5.6 B & D). For total plant length, KAfr were higher than KAc, while both these treatments did again not differ from control plants (fig. 5.6 G). The accumulation of root dry weight was inhibited by exposure to control light grown Alva plants relative to plants that were not exposed to volatiles from other plants, and this effect was lost when Alva emitters were exposed to low R:FR conditions. (fig. 5.6 H). In addition KAfr had a higher total root length than did KAc (fig. 5.6 I). Although KAfr and KAc differed in total leaf area and leaf dry weight (fig. 5.6b & c), the SLA was found to be similar for all treatments (fig. 5.6 J). The shoot mass fraction (SMF, g shoot g-1 plant) was highest in KAc (fig. 5.6 K), indicating an increased dry mass allocation to shoots of Kara plants in response to control light-grown Alva VOCs. Finally the LMF was higher in KAfr than KAc and not different between KAc and control Kara plants that were not exposed to Alva at all (fig. 5.6 L). It is noteworthy that for most parameters, Kara plants that were not exposed to an Alva neighbour at all were not significantly different from either KAc or KAfr, except for root dry weight and LMF (fig. 5.6 H & L).

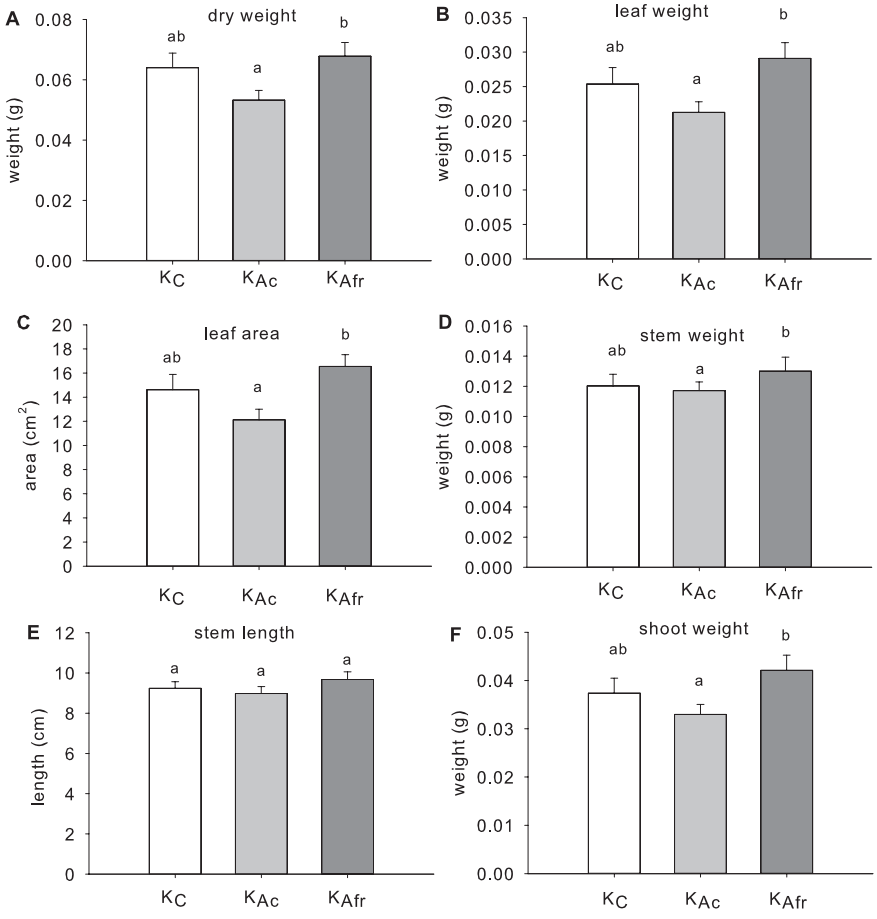
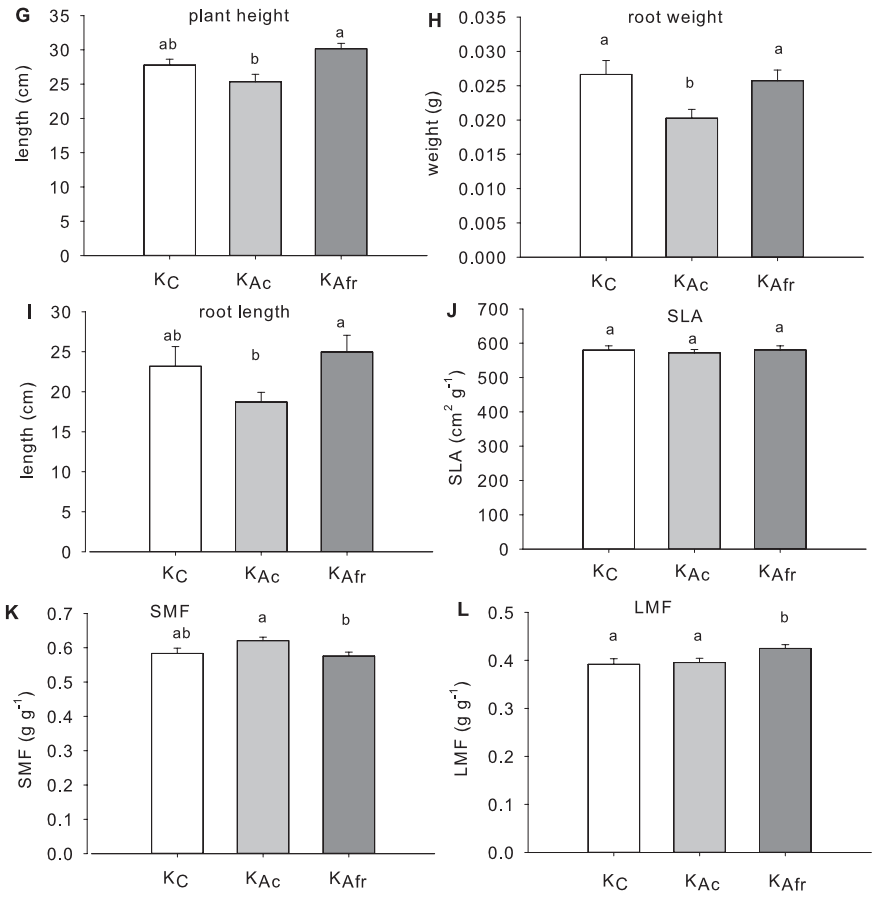


Figure 5.6 Growth response of Alva plants after 10 day exposure to low R:FR conditions (low R:FR) or under control light conditions (control). In panel C, leaf mass fraction (LMF, g leaf g-1 plant) is shown. Panel d represents specific leaf area (cm2 leaf g-1 leaf) Bars represent means ± SE (n=18-19). Data were analysed with a Student's t-test. Significant differences are indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

Figure 5.6 Continued



Ethylene exposure reduces plant height and leaf area of Kara plants

Since figure 5.3 showed that ethylene production by Alva plants is enhanced by low R:FR conditions, it was tested whether differences in resource allocation between KAc and KAfr could be due to differential ethylene emissions by Alva plants. To this end, Kara plants, exposed to air containing 25 and 230 ppb ethylene, were compared with control plants. No effect of ethylene exposure was found on total dry weight, or dry weight of leaves, stems or roots (fig. 5.7 A-D). However, total plant height of Kara exposed to 230 ppb ethylene was reduced compared to control plants (fig. 5.7 E), although no differences in stem length were observed (fig. 5.7 F). Both 25 and 230 ppb ethylene led to reduced leaf area compared to control plants (fig. 5.7 G), but no differences in the SLA were found (fig. 5.7 H). In contrast to the observed differences in SMF and LMF between KAc and KAfr (fig. 5.6 K & L), no ethylene-induced differences in LMF were found (fig. 5.7 I & J).

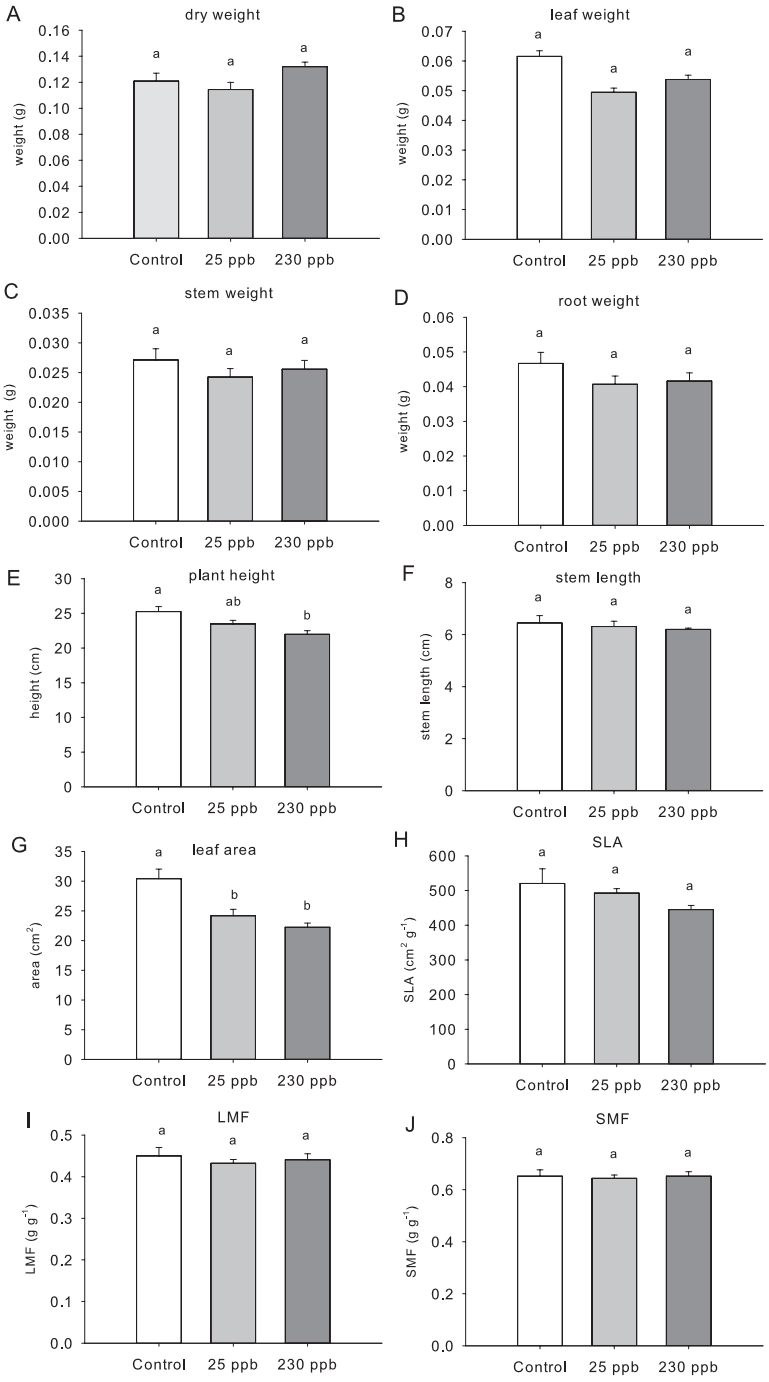


Figure 5.7 Growth response of Kara plants after 10 d exposure to 0, 25 or 230 ppb ethylene. Bars represent means ± SE (n=12). In H-J, specific leaf area (SLA), leaf mass fraction (LMF) and shoot mass fraction (SMF) are shown. Significant differences are indicated with different letters (one-way ANOVA, Bonferroni post hoc test, alpha = 0.05).

Discussion

This study demonstrates that exposure of the barley cultivar Alva to low R:FR conditions results in a reduced total emission of VOCs. In addition, also the relative composition of the VOC blend is affected by low R:FR since the emission of some components is reduced, while the emission of other compounds is not affected or even induced. Coinciding with these changes in volatile emission, exposure of the Kara cultivar to VOCs of low R:FR exposed Alva plants no longer led to increased shoot mass fraction or reduced root dry weight. Moreover, Kara plants exposed to VOCs of low R:FR-grown Alva plants accumulate more shoot dry weight and has a higher LMF than Kara plants exposed to Alva plants grown under normal light.

In the previous chapter, it was demonstrated that both constitutive and methyljasmonate induced volatile emission of *Arabidopsis thaliana* are reduced under low R:FR. In the current chapter it is demonstrated that exposure to low R:FR also reduced the emission of several VOCs in barley, suggesting that reduced volatile emission in low R:FR conditions might be widely spread in plant species. Three out of six significantly down-regulated Alva VOCs are sesquiterpenes. Emission of the sesquiterpene (*E*)-β-caryophyllene has been shown to be sensitive to changes in light intensity and temperature in maize (*Zea mays*) (Gouinguene & Turlings, 2002). The data provided here indicate that the emission of β-caryophyllene is also reduced under low R:FR conditions, thus adding light to the environmental parameters that control the emissions of this volatile sesquiterpene. However, both these data as well the previously published data on light intensity and temperature (Gouingene & Turlings, 2002) do not address the question whether the observed changes in β-caryophyllene emission are due to environmental control of β-caryophyllene biosynthesis, volatilization, stomatal conductance or still other factors that influence VOC emission. Since the emission of only specific VOCs are reduced by low R:FR it is likely that the effects on sesquiterpenes are not related to stomatal conductance or other factors that would generically affect emissions of all VOCs.

Although the majority of VOCs was not affected or suppressed by low R:FR, the emission of two compounds was clearly enhanced: linalool oxide and ethylene. The enhanced ethylene emission of Alva is in accordance with enhanced ethylene production of low R:FR-exposed sorghum (*Sorghum bicolor*) (Finlayson et al., 1999), Arabidopsis (Pierik et al., 2009) and many other species (reviewed in Kegge & Pierik, 2010).

In cultivated tobacco (*Nicotiana tabacum*), it has been suggested that perception of ethylene levels that accumulated in dense stands (approximately 25 ppb) may serve as a neighbour detection cue (Pierik et al., 2004b). We show here that low R:FR exposure also induces ethylene emission of the Alva cultivar, but the

absolute ethylene emission rate by barley is very low compared to other species. Low R:FR-exposed *A. thaliana*, for example, produced up to 0.10 nmol ethylene g-1 fresh weight h-1 (fig. 3.2 A), while barley produced no more than 0.012 nmol ethylene g-1 fresh weight h-1 (fig. 5.3). Therefore, in cuvettes with Kara receivers, ethylene levels were unlikely to reach concentrations that are physiologically meaningful. Nevertheless effects of elevated ethylene levels were investigated. While ethylene-exposed plants showed reduced length, Kara plants exposed to low R:FR grown Alva VOCs (KAfr) were longer than Kara plants exposed to normal light grown Alva (KAc). This argues against a role for ethylene in Kara responses to low R:FR induced ethylene production in Alva. The observations that exposure to high levels of ethylene did not affect shoot and root mass fraction, nor root length or weight (fig. 5.7) further argues against an important role for ethylene in affecting biomass allocation in Kara in response to air derived from Alva plants.

The consequences of modulation of VOC emission by low R:FR exposure were studied in neighbouring plants exposed to these VOCs. It was previously shown that the Kara cultivar shows changes in carbon allocation upon exposure to the VOC blend emitted by Alva cultivar neighbours (Ninkovic, 2003), whilst not responding to VOC blends from conspecific Kara neighbours. Here, we confirm that Alva VOCs affect carbon allocation in Kara neighbours. Notably, the shoot mass fraction increased and root weight decreased in Kara exposed to control light-grown Alva as compared to plants that were not exposed to neighbours. These effects are, however, partly different from those observed by Ninkovic (2003). Ninkovic demonstrated that Kara plants exposed to volatiles derived from Alva neighbours invested more in root biomass and had an increased SLA. Since the same cultivars have been used in both studies, we speculate that these differences between the two studies are related to major differences in growth conditions. The current experiments used plants grown in growth chamber conditions and with a light-impenetrable separation between the receiver and emitter plants. The experiments of the study by Ninkovic on the other hand, were collected in a greenhouse with higher and less controlled light intensities and with light reflection between emitter and receiver plants being possible.

The Kara plants exposed to VOCs emitted by Alva plants grown under control light conditions had lower total dry weight (fig. 5.6 A) than those exposed to VOCs of low R:FR-grown Alva plants, but invested more of their total biomass in shoot organs (higher SMF; fig. 5.6 K). This is striking given that SMF was previously shown to increase with size in this barley cultivar (Ninkovic, 2003). Thus, the increased allocation to shoot organs in KAc plants is unlikely a consequence of their lower total plant mass. Put differently, the observed changes in allocation in response to VOCs emitted by neighbours are likely truly plastic changes and not the consequence of putative ontogenetic effects on allometry.

The increase in SMF in KAc is also what can be observed at increasing plant den-

sities (Poorter et al., 2012). Because in dense stands, some VOCs may still accumulate to sufficiently high levels to exert effects on neighbours before shading would reduce their emissions, it would still be possible that Kara plants respond to Alva VOCs as a cue of neighbour proximity. Enhanced SMF would allow for optimizing aboveground competitive performance, for example by investing this carbon in stems that allow a plant to grow taller and reach the light: the shade avoidance syndrome.

However, stem length did not increase in KAc, compared to other treatments (fig. 5.6 E), implying that the increase in SMF in KAc is not a classic induction of shade avoidance. Moreover, the leaf mass fraction also increases in KAfr compared to both unexposed Kara plants and Kara plants exposed to VOCs from control Alva plants. A meta-analysis on many different species showed that plants under low R:FR conditions typically show a mild decrease of LMF (Poorter, 2001; Poorter et al., 2012), probably associated with the strongly increased investments in stem growth. The observed reduction in total dry weight in KAc compared to KAfr could imply that (compounds in) the VOC blend derived from Alva neighbours are allelopathic to Kara plants. This reduction in total plant dry weight in KAc is caused by a reduction in dry weight of root, stem and leaf. Monoterpenes produced by *Salvia leucophylla* can inhibit root growth in *Brassica campestris* (Nishida et al., 2005) through inhibition of cell proliferation in the root apical meristem. Possibly, (one of) the compounds released by Alva plants under the current experimental conditions inhibit growth in Kara plants. Although these VOC-mediated effects of Alva on Kara plants may impact on Kara competitive performance, these effects are negated by the exposure of Alva plants to low R:FR conditions. It has been demonstrated that volatiles of several barley cultivars can reduce aphid settling on receiver barley plants (Petterson et al., 1999), suggesting that plant-plant signalling between different barley cultivars is not restricted to Alva and Kara plants. We, therefore, expect that the effects of (low R:FR treatment on) volatile exposure to resource allocation is not restricted to the studied Alva-Kara system, but more widely spread through barley cultivars and possibly other species.

It is important to realize that under high density agricultural field conditions plants will always be exposed to low R:FR light conditions, at least in the horizontally reflected light. Although these conditions are likely to directly affect carbon allocation in Kara plants, they certainly have a major impact on VOC emissions of Alva plants, and the biological effects of these VOCs on exposed Kara neighbours.

In conclusion, we demonstrate that low R:FR exposure of Alva plants changes its emission of VOCs. This finding is in agreement with data on Arabidopsis where low R:FR also affected the emissions of various VOCs and in both species the total VOC emission is reduced in low R:FR. We showed previously in Arabidopsis that these light quality effects have implications for VOC-mediated plant-herbivore interactions and the current data show that these R:FR effects on VOC emissions also affect VOC-mediated plant-plant information transfer.

Acknowledgements

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CHAPTER

1 2 3 4 5 6

Summarizing discussion

Plants are challenged throughout their life cycle by changes in their environment that can reduce plant fitness. In order to survive and perform well, plants need to acclimate to these environmental challenges, which include for example changes in meteorological conditions, competing neighbouring plants, pathogens and herbivorous insects. Plant species have evolved specialized traits to deal with specific environmental stresses. From an ecophysiological perspective, intriguing questions to answer are i) how do plants perceive and process the cues from their environment that indicate certain stresses?, and ii) how do plant responses to one cue affect responses to co-occurring cues ? This thesis focused on plant growth in dense stands and asks which above-ground cues occur in dense stands, which of these cues are involved in early neighbour-detection and what are their effects on plant growth in dense stands. Specific emphasis is on the emission of volatile organic compounds (VOCs).

Successive cues in dense stands

At different stages of a closing canopy, plants perceive different cues from neighbouring plants. A summarizing scheme of these different cues is presented in fig. 6.1. It is generally accepted that a reduction in the ratio between red (R) and far-red (FR) light is the first cue that plants use to detect nearby neighbours. This R:FR reduction is caused by early horizontal reflection of FR light by neighbouring plants. However, if plants grow in a flat canopy, such as formed by rosette plants, there will hardly be any horizontal reflection due to the lack of height growth and it was shown in chapter 2 that in such stands the R:FR does not change as much as has been described for stands of vertically growing plants. It was also shown in chapter 2 that touching of leaf tips of surrounding neighbours constitutes the earliest neighbour detection mechanism in the rosette species Arabidopsis (*Arabidopsis thaliana*). Touching leaf tips lead to an increased petiole angle (i.e. a more vertical orientation of the leaves), which stimulates a plant's competitive position in a dense stand. Interestingly, hyponasty also creates a vertical canopy structure that does have the potential to reflect FR light, thereby creating a reduction in the R:FR conditions of horizontally reflected light (chapter 2). This reduction in the R:FR ratio subsequently leads to enhanced petiole elongation, further increase of petiole angle (hyponasty) and enhanced emissions of the gaseous plant hormone ethylene. Ethylene has been previously associated with shade avoidance and plant competition for light (Pierik et al., 2003, 2004b). In chapter 3 it was shown that indeed in Arabidopsis stands ethylene perception is involved in the timing of shade avoidance and is essential for petiole elongation induced by the low R:FR conditions that occur as a consequence of the earlier mentioned touch-induced

hyponastic leaf growth. When the canopy continues to develop true leaf overlap will occur leading to reduced levels of blue light and photosynthetically active radation (PAR). These signals themselves can further induce shade avoidance responses (e.g. Pierik et al, 2004a; Keller et al., 2011; Keuskamp et al., 2011) and may also stimulate low R:FR-induced responses (De Wit, 2012).

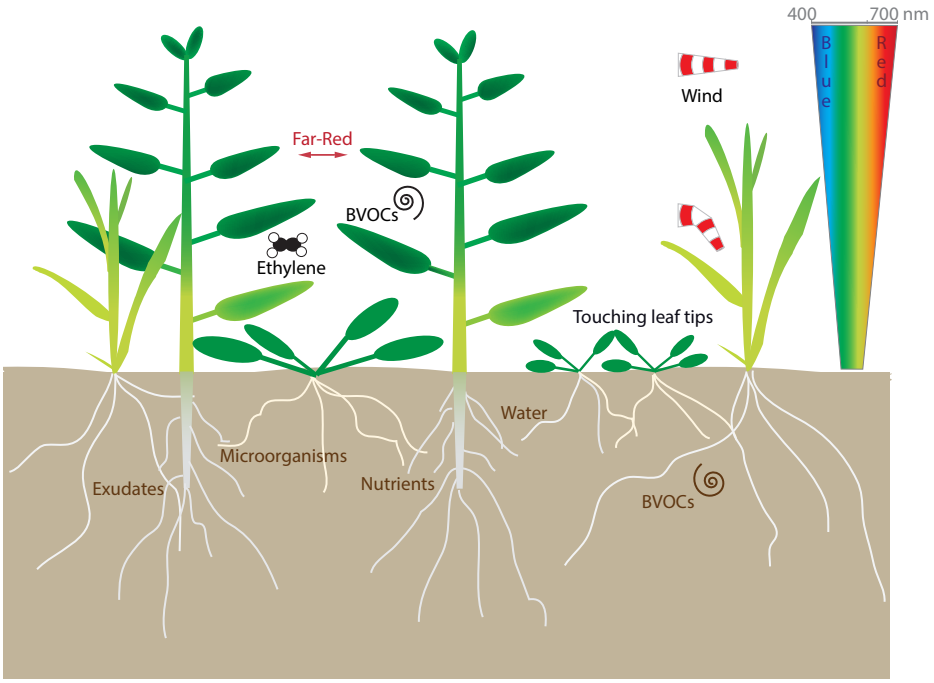

Figure 6.1 Detection of neighbouring competitors by plants in dense vegetation. Blue and Red (R) light are selectively absorbed by chlorophyll for photosynthesis, whereas Far-red (FR) light is mostly reflected by the leaves. Plants sense reduced blue light fluence rates using the cryptochrome and phototropin families of photoreceptors, whereas R:FR is monitored using the phytochrome family of photoreceptors (Franklin, 2008; Vandenbussche et al., 2005). Ethylene emission is induced under shading conditions (chapter 4) and can serve as a volatile cue to detect neighbours in a canopy (Pierik et al., 2004). Plant responses to reductions in R:FR are modulated by ethylene perception (chapter 3). The emission of VOCs other than ethylene is partially reduced under shading conditions (chapter 4 & 5) and this affects information transfer through volatiles between plants (chapter 5). The reduced wind exposure inside dense stands might also act functionally as an indicator of dense vegetation (Anten et al., 2005). We demonstrate that touching leaf tips are involved in inducing hyponasty in early neighbour detection in Arabidopsis (chapter 2). This response to touch differs from mechanical stress as described in literature (Anten et al., 2005, 2006; Braam, 2005). Belowground neighbour detection can occur through neighbour-induced changes in resource availability, such as local water and nutrient depletions (de Kroon, 2007), through root exudates (Bais et al., 2006) and can even be affected by soil microorganisms (Raaijmakers et al., 2009). Plants can also negatively affect neighbours by exuding allelopathic compounds that inhibit growth or seed germination (Bais et al., 2006).

Blue light depletion and low R:FR conditions partly converge on shared regulators, such as Phytochrome Interacting Factors (PIFs), auxin and brassinosteroids (Keller et al., 2011; Keuskamp et al., 2011; Pierik et al., 2009) and ethylene also interacts with specific PIF proteins (Zhong et al., 2012), auxin (Pierik et al., 2009) and brassinosteroids (Polko et al., in press). It is, therefore, possible that the perception of different neighbour detection cues converges on a shared molecular module of growth control.

Volatile signalling in dense stands

For the perception of physiologically relevant concentrations of VOCs emitted by neighbouring plants, small inter-plant distances are required (Heil & Adame-Alvarez, 2010; Frost et al., 2008; Karban, 2007; Karban et al., 2003), which typically occur in dense stands. In chapters 4 and 5, effects of neighbour-related shading conditions that occur in dense stands on the emission of (methyl jasmonic acid (MeJA)-induced) VOCs were investigated. Both constitutive and MeJA-induced VOC emissions were found to be reduced under low R:FR, shade and dense canopy conditions. Reduced emission of MeJA–induced VOCs is in accordance with the FR-regulated suppression of JA-mediated direct plant neighbour in various species (e.g. Izaguirre et al., 2006; Moreno et al., 2009; De Wit, 2012). This low R:FR-mediated suppression of neighbour is most probably associated with reduced responsiveness to JA by enhanced expression of JASMONATE ZIM-domain protein *JAZ10* (Cerrudo et al., 2012; Ballaré et al., 2012), which is a negative regulator of JA signalling. As a result of reduced total VOC emission and/or changed composition of VOC blends under low R:FR conditions, plant preference of the specialist herbivore *Pieris brassicae* changed. *P. brassicae* preferred MeJA-treated plants over control plants when both groups were grown under control light conditions. However, this preference disappeared when both control and MeJA-treated plants were grown under low R:FR conditions (chapter 4). These data indicate that FR signalling by plants in dense stands cascades into higher trophic levels, such as herbivores, through effects on VOC emissions. VOC emissions play a significant role in indirect plant defences by attracting/repelling other organisms (reviewed by Dicke & Baldwin, 2010). JA-treated plants, for example, are less attractive to butterflies for egg deposition (Bruinsma et al., 2007) and also predators of herbivorous insects can use VOCs emitted by herbivore-attacked plants to locate the herbivores (e.g. Muller & Hilker, 2000). This induced emission has also been coined “a cry for help” and the attracted predators essentially serve as “bodyguards” for the attacked plants that emit the VOCs (Dicke, 2009; Dicke & Sabelis, 1987). It is possible that growth at high densities interacts with, and possibly even compromises these multitropic interactions, due to the pronounced effects of light quality and quantity on emission of specific VOC components.

Although the effect of light quantity on VOC emission was previously published

(e.g. Gouingene et al., 2002), it had not been demonstrated that neighbour-related changes in light quality conditions also affect the emission of VOCs (chapters 4 & 5). The studies on barley, presented in chapter 5 indicate that resource allocation of plants exposed to VOCs from emitter plants grown in low R:FR conditions was different from plants exposed to emitter plants grown in control light conditions (chapter 5). Barley receiver plants, grown in control light conditions, exposed to VOCs from emitter plants grown in control light conditions, developed, amongst others, a higher shoot mass fraction than barley receiver plants exposed to VOCs of low R:FR grown plants.

Typically, low R:FR exposed plants are known to increase their relative investment in shoot biomass as was shown for soybean by Kasperbauer (1987) and Kasperbauer & Hunt (1992) and constitutive shade avoiding *hy3* mutants in Arabidopsis also increase relative investment in shoot biomass (Reed et al., 1993). It thus appears that exposure of barley plants to VOCs of a non-FR receiving neighbour induces similar changes in allocation as does direct exposure to low R:FR conditions. Interestingly, this effect disappears when the VOC-emitting plant is exposed to low R:FR conditions, but most probably the receiver will by then also be exposed to these reduced R:FR conditions. It would be interesting to study whether initial exposure to this VOC blend, followed by exposure to low R:FR conditions which follow when the growing canopy closes, would lead to enhanced shade avoidance responses compared to low R:FR exposure alone.

It is at present not clear whether the altered carbon allocation of barley upon volatile exposure has a fitness benefit for the VOC emitter, receiver, both or none. Volatile signalling can be beneficial for emitter plants if VOC components are allelopathic to neighbours, but in other cases, the VOC-sensing neighbours can exploit the perception of these VOCs (e.g. Karban et al., 2003, Kessler et al., 2006). In cases where a receiver profits from information generated by the emitter, without returning a benefit to the emitter, plant volatile information transfer is referred to as “eavesdropping” (Dicke et al., 2003; Karban et al., 2003; Heil & Karban, 2010). The modified emission of VOCs by exposure to low R:FR conditions might have a broad impact on ecological systems: the occurrence of low R:FR conditions is very widespread and occurs in all dense stands of plants and VOCs are known to mediate within-plant and inter-plant interactions, to function as priming agents for direct and indirect plant neighbours and as attractant for carnivores, herbivores or pollinators (e.g. Bruinsma et al., 2007; Muller & Hilker, 2000; Dutton et al., 2000).

It is of great interest for agricultural systems, in which cultivar mixtures or intercropping systems are used, to investigate whether VOC-dependent eavesdropping or plant-insect interactions are affected when VOC emitting plants are exposed to low R:FR conditions. Neighbour-related changes in light conditions have an effect on interactions between plants and insects (chapter 4) and between different barley cultivars (chapter 5). Cultivar mixtures or intercropping

systems may be less effective in suppressing for example herbivore proliferation than anticipated from low density or single plant experiments. Since the emission of VOCs is reduced in dense stands, it is likely that various modes of information transfer through VOCs are affected by changed emissions. For example, within- and between-plant information transfer, such as priming for defence through volatile compounds, might be affected by reduced VOC emissions from plants in dense stands. Also predators of herbivorous insects that make use of volatile cues might be less efficient in dense stands in finding infested plants, resulting in more plant damage than expected from studying individually growing plants.

Future perspectives

By demonstrating touching leaf tips as a new neighbour detection cue and showing that changes in light quality affect the emission of VOCs and their impact on plant-plant interactions, this thesis aims to contribute to more in-depth insight into plant-plant interactions. Future research could be focused towards different directions: e.g. identifying regulatory mechanisms and determining the impact of our findings at larger, ecological and agronomical scales. A summarizing scheme of known regulatory mechanisms is presented in fig. 6.2. In this thesis, we were able to demonstrate that touching leaf tips can be used as a cue to detect neighbours and that reductions in the R:FR ratio negatively affect the emission of (MeJA-induced) VOCs. The molecular mechanisms underpinning the touch-induced hyponastic response in early neighbour detection are not understood, even though molecular mechanisms of responses to other forms of mechano-stimulation have been unraveled to some extent (Braam, 2005; Chehab et al., 2012). Also, it remains unknown how phytochrome modulates the emission of some, but not all, VOCs. Although some knowledge is available on how low R:FR conditions desensitize plants to JA by inducing the *JAZ10* expression (Moreno et al., 2009; Cerrudo et al., 2012), it remains to be studied whether this is also the mechanism through which MeJA-induced VOC emissions are suppressed. Furthermore, it remains unknown how low R:FR-induced phytochrome inactivation leads to suppression of constitutively emitted VOCs. Since both the model plant Arabidopsis and the cereal crop barley show a similar reduction in the emission of VOCs under low R:FR conditions, knowledge on the mechanisms underpinning these responses in Arabidopsis would be of considerable importance in agriculture to create, for example, varieties that do not show a FR-mediated suppression of VOCs.

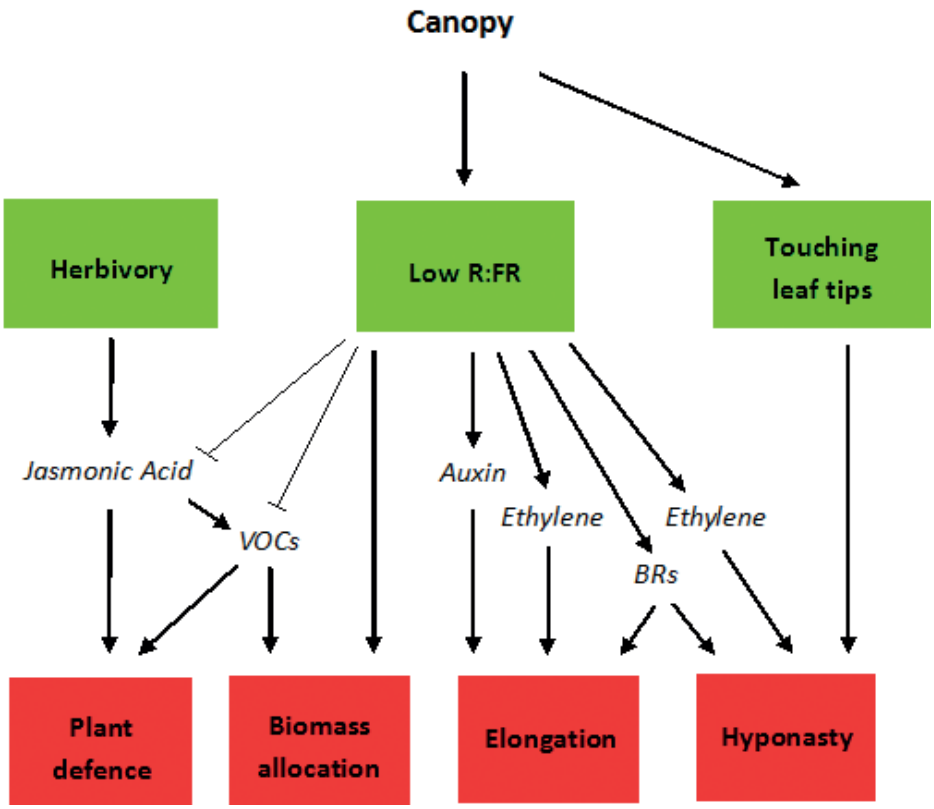

Figure 6.2 Scheme of regulatory mechanisms of cues in dense stands. In dense plant canopies, reduced Red:Far-red (R:FR) ratio's and touching leaf tips can serve as neigbour detection cues. Touching leaf tips can lead to increased hyponasty, but the molecular mechanism underpinning this response remains to be unraveled. Reduced R:FR conditions can lead via several hormonal pathways to increased petiole elongation and hyponasty. Low R:FR induced hyponasty and petiole elongation are modulated by ethylene and low R:FR-dependent elongation is also under control of auxin and brassinosteroids (BRs). Besides regulation of the shade avoidance response, changes in R:FR conditions also affect plant defense and the emission of (MeJA-induced) VOCs. Reduced R:FR negatively affects JA-dependent plant defense and reduce the emission of MeJA-induced VOCs. These reduced emission of VOCs under low R:FR conditions modify effects of volatiles on biomass allocation in neighbouring plants.

In dense stands of plants with a vertical growth structure, like barley or tomato, not all leaves are equally shaded. Lower leaves are fully shaded, whereas other leaves perceive a reduction in R:FR conditions and the higher leaves in a plant might even be unaffected. In chapter 4, we demonstrate that exposure to low R:FR conditions was sufficient to suppress (MeJA-induced) VOC emissions. Since herbivory is known to affect volatile emissions both locally and systemic (Heil & Bueno, 2007; Dicke & Baldwin, 2010), it would be interesting to study whether the effect of shade on the emission of (MeJA-induced) VOCs is acting local or systemic and if there is a difference in low R:FR-mediated suppression of local verus systemic MeJA-induced VOCs. Information transfer through VOCs

can both function within or between plants (e.g. Heil & Bueno, 2007; Karban, 2003). The reduction of VOC emissions in low R:FR conditions is shown to affect plant-plant interactions (chapter 5) and it would be of interest to investigate whether neighbour-related changes in light quality also affect within-plant volatile signalling, especially taking into account the question whether shade effects on plant volatile emission are local or systemic.

It is generally meaningful to verify the importance of data such as presented in this thesis under natural conditions and for a wider set of plant species. For example, the response we observed in Arabidopsis on touching leaf tips might be less crucial in early neighbour detection in non-rosette species that form vertical canopies. Nevertheless, plants of any growth form will physically interact above and below ground, with nearby neighbours in dense stands. It is, therefore, likely that these mechanical interactions, together with the plethora of other cues that occur in dense stands, co-determine plant morphology and growth.

It is, however, important to realize that the hormonal network and physiological abilities of Arabidopsis to respond to environmental stresses are largely dependent on developmental stage (Diezel et al., 2011) and therefore, it would be interesting to verify whether responses to touch in dense stands are constant through plant development. The effect of low R:FR conditions on the emission of VOCs was found in barley and in Arabidopsis, a mono- and dicot plant, which is an indication that this effect could be widely dispersed throughout the plant kingdom. It is, however, possible that species that differ in the way in which they process light cues, would also differentially affect VOC emissions. It would, therefore, be of interest to investigate VOC emission of non-shade avoiding, for example shade-tolerant, species.

This thesis is one step towards a better understanding of information transfer between plants in dense stands. In order to advance our understanding of interactions between plant responses to different cues that are generated in dense stands, it is of importance to combine knowledge on the different ways in which plants perceive their environment and study the regulatory mechanisms that are controlling the perception of environmental changes in response to the multitude of cues that exist in dense, competitive stands.

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Samenvatting in het Nederlands

Om te kunnen groeien hebben planten licht, water en voedingsstoffen nodig. Als planten in een omgeving groeien waarin ook andere planten groeien, dan vindt er concurrentie plaats om licht, water en voedingsstoffen. Bij concurrentie voor deze benodigdheden is het van belang om naburige planten tijdig waar te nemen en op hun aanwezigheid te kunnen reageren. Op deze manier kunnen planten ervoor zorgen dat ze beschikken over voldoende licht, water en voedingsstoffen, desnoods ten koste van andere planten.

Dit proefschrift beperkt zich tot bovengrondse interacties tussen planten. Bovengronds concurreren planten om licht. Planten absorberen vooral rood en blauw licht voor fotosynthese en reflecteren groen en ver-rood licht. Planten danken hun groene kleur aan de reflectie van groen licht. De verandering in de verhouding tussen rood en ver-rood licht nemen planten waar met specifieke lichtreceptoren die phytochromen genoemd worden. Een daling van de relatieve hoeveelheid rood licht en een stijging van ver-rood licht zijn voor de plant een signaal dat er andere planten in de directe omgeving zijn. Hierop kan een plant reageren door zich te strekken, zijn bladeren meer omhoog te richten (dit wordt hyponastie genoemd) en in sommige gevallen zelfs vroeger te bloeien.

In hoofdstuk 2 van dit proefschrift is onderzocht hoe in *Arabidopsis thaliana* (zandraket) planten elkaar boven de grond waarnemen. Uit experimenten bleek dat het aanraken van de toppen van bladeren van naburige planten het eerste signaal is dat leidt tot een hyponastische respons. Deze aanraking hoeft niet per se van een andere plant te zijn. Planten reageren op vergelijkbare wijze als ze bijvoorbeeld tegen lichtdoorlatende plastic stekers aangroeien. De resultaten tonen aan dat planten elkaar al kunnen waarnemen, voordat er verandering in lichtsamenstelling optreedt. Met behulp van een computermodel dat de lichtreflectie uitrekent, werd eveneens aangetoond dat in dit vroege stadium de verandering in verhouding tussen rood en ver-rood licht niet groot genoeg is om voor hyponastie of extra strekking van de bladstelen te zorgen.

Het plantenhormoon ethyleen staat bekend om zijn rol in de rijping van fruit. Dit plantenhormoon is gasvormig en wordt daarnaast ook geassocieerd met plantengroei, phytochroom interacties en het waarnemen van naburige planten. In hoofdstuk 3 werd de interactie tussen dit hormoon en het vermogen van de plant om te reageren op veranderingen in de rood : ver-rood verhouding onderzocht. Het onderzoek liet zien dat ethyleen-ongevoelige planten geen extra strekking van bladstelen vertonen als de verhouding van rood : ver-rood licht licht verandert, zoals het geval is als planten in een vegetatie nog niet zo dicht naar elkaar toe gegroeid zijn. Hieruit blijkt dat ethyleen belangrijk is in het regelen van deze strekking. Bij een sterkere verandering in de rood : ver-rood verhouding strekken de

bladstelen van ethyleen-ongevoelige zandraket op een vergelijkbare wijze als de bladstelen van ethyleen gevoelige planten. Echter, de hyponastische respons als reactie op een verandering in de rood : ver-rood ratio van ethyleen-ongevoelige planten komt langzamer op gang dan in ethyleen gevoelige planten.

Competitie voor licht zorgt er niet alleen voor dat planten investeren in strekkingsgroei waardoor ze mogelijk uit de schaduw van hun concurrenten weg groeien, maar heeft ook effect op processen in planten die niet direct gerelateerd zijn aan competitie voor licht. Zo is het bekend dat planten in de schaduw vatbaarder zijn voor diverse ziektes en minder goed in staat zijn om zichzelf te beschermen tegen insecten, zoals rupsen. Als een plant aangevreten wordt door een insect, dan produceert de plant vluchtige plantenstoffen en stoffen die schadelijk zijn voor de insecten. De vluchtige stoffen kunnen dienen als signaal voor naburige planten en ook voor de natuurlijke vijanden van het insect dat de plant aanvreet. In hoofdstuk 4 is onderzocht hoe de productie van vluchtige stoffen verandert wanneer een plant in de schaduw staat. Van verschillende vluchtige stoffen bleek de hoeveelheid die een plant in de schaduw uitstoot lager dan de hoeveelheid in normaal licht. Ook de uitstoot van sommige vluchten stoffen die geïnduceerd worden door een behandeling met een plantenhormoon (methyl jasmonaat) dat betrokken is bij de afweer tegen insecten, is verminderd als planten blootgesteld zijn aan een lage rood : ver-rood verhouding. Doordat zandraker vluchtige stoffen in een andere samenstelling produceert wanneer ze onder een verlaagde rood : ver-rood verhouding zijn opgegroeid, zijn de rupsen van het grote koolwitje (*Pieris brassicae*) niet meer in staat om onderscheid te maken tussen planten die wel en niet behandeld zijn met methyl jasmonaat.

Hoofdstuk vijf laat zien dat een verandering in de rood : verrood verhouding niet alleen in zandraket tot een vermindering van de emissie van vluchtige stoffen leidt, maar ook in gerst (*Hordeum vulgare*). Van gerst is bekend dat de vluchtige stoffen van het ras Alva effect kunnen hebben op de biomassa verdeling tussen het wortelstelsel en de scheut in het ras Kara. De data in dit proefschrift laten zien dat Kara groter was, langere stengels en wortels had en grotere en zwaardere bladeren als ze blootgesteld werden aan vluchtige stoffen van Alva planten die opgegroeid waren in licht met een lage verhouding rood : ver-rood licht, in vergelijking met Kara planten die blootgesteld werden aan vluchtige stoffen van Alva planten die opgegroeid waren in licht met een hoge verhouding rood : ver-rood licht. Daarnaast hadden deze Kara planten relatief grotere wortels en bladmassa. Dit proefschrift laat zien dat aanraking de eerste manier is waarop planten hun directe buren waarnemen. De respons op deze aanraking leidt tot veranderingen in de verhouding tussen rood en ver-rood licht. Het gasvormige plantenhormoon ethyleen is betrokken bij de regulatie van de respons op veranderingen in verhouding tussen rood en ver-rood licht. Dit proefschrift laat verder zien dat ethyleen de hyponastische respons wel moduleert, maar geen noodzakelijk voorwaarde

is voor de reactie om überhaupt op te treden. Als planten beschaduwd worden produceren ze minder vluchtige stoffen. Deze verminderde productie heeft tot gevolg dat de impact van deze vluchtige stoffen op hun omgeving verminderd is.

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Curriculum Vitae

Wouter Kegge werd geboren op 10 februari 1984 te Gouda. In 2002 behaalde hij zijn Gymnasium diploma aan het Minkema College in Woerden.

In september 2002 startte Wouter bij de Universiteit Utrecht met de bacheloropleiding Biologie. Tijdens het studiejaar 2003-2004 zat Wouter in het bestuur van de Utrechtse Biologen Vereniging. In 2006 behaalde hij zijn bachelordiploma en begon aan de masteropleiding Plant Biology. Zijn eerste onderzoeksstage werd uitgevoerd onder begeleiding van Jurriaan Ton en Marieke van Hulten. In de vakgroep fytopathologie hield Wouter zich bezig met de karakterisering van een mutant die geen geïnduceerde resistentie vertoonde na behandeling met BABA. De tweede onderzoeksstage voerde hij uit in het lab van prof. Farmer in Lausanne. Onder begeleiding van dr. Reymond deed Wouter onderzoek naar de rol van g-eiwitten bij de afweer van planten tegen rupsenvraat van de generalist *Spodoptera exigua*.

Vanaf september 2008 tot november 2012 deed Wouter promotieonderzoek bij de vakgroep " Ecofysiologie van Planten" aan de Universiteit Utrecht. Daar werd onder begeleiding van dr. R. Pierik, prof. dr. L.A.C.J. Voesenek en prof. dr. M. Dicke het onderzoek uitgevoerd dat beschreven staat in dit proefschrift.