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Population level effects of green leaf volatiles and ethylene production in seedlings

Bachelorthesis

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2. Index of Abbreviations

ACC	aminocyclopropanecarboxylate
ACO	aminocyclopropanecarboxylate oxidase
ACCS	aminocyclopropanecarboxylate synthetase
BVOC	biogenic volatile organic compounds
cDNA	complementary deoxyribonucleic acid
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EI	electron impact
ET	ethylene
EV	empty vector line
FR	far-red light
GA ₃	gibberellic acid
GC-QMS	gas chromatography - quadrupole mass spectroscopy
GLVs	green leaf volatiles
13HPOT	13-hydroperoxide
irACO	inverted repeat <i>aminocyclopropane-carboxylate oxidase</i> line
IRGA	infra-red gas exchange analyzer
irLOX2	inverted repeat <i>lipoxygenase 2</i> line
JAs	jasmonates
JA	jasmonic acid
LAE	leaf area expansion
LED	light-emitting diode
<i>NaACO</i>	<i>Nicotiana attenuata aminocyclopropane-carboxylate oxidase</i> gene

<i>NaLOX2</i>	<i>N. attenuata lipoxygenase 2 gene</i>
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
Pfr	activated phytochromes
Photo	photosynthesis
PIF	phytochrome interacting factors
Pr	inactivated phytochromes
PTFE	polytetrafluoroethylene
qPCR	quantitative polymerase chain reaction
R:FR	red:far-red ratio
R	red light
RNA	ribonucleic acid
SAM	S-adenosyl methionine
TAE buffer	TRIS-acetate-EDTA buffer
TD-GC-MS	thermal desorption - gas chromatography - mass spectroscopy
Trans	transpiration

3. Introduction

3.1 Overview of plant neighbor sensing

In large plant populations there occurs mostly limitation of light, water or nutrient sources. As a consequence, plants have to compete with neighbors to ensure their survival. Plants have different abilities to detect and respond to proximate competitors. Competition can occur both below- and aboveground (Casper & Schenk, HJ, Jackson, 2003; Ronald Pierik & de Wit, 2013). Indicators for proximate neighbor plants are light

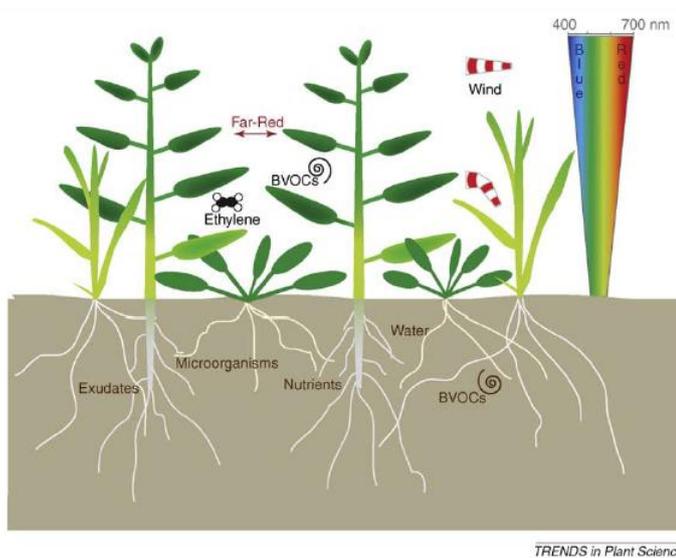


Figure 1: Detection of neighboring competitors by plants in dense vegetation. Picture is taken from (Kegge and Pierik 2010).

quality, wind, microorganisms, lack in nutrients or water, as well as chemical substances, like ethylene, exudates or biogenic volatile organic compounds (BVOCs, Figure 1). As a result, plants respond with several phenotypical modifications to increase their fitness. For instance, belowground a lack of water or nutrients, such as nitrate, ammonium or phosphate, would induce so-called “nutrient foraging” (Ronald Pierik, Sasidharan, & Voeselek, 2007). This term refers to rapid development of root length and density into nutrient-rich patches. A second possible belowground competition response is allelopathy, in which plants produce and emit allelochemicals that accumulate in soil and suppress seed germination, growth and establishment of potential competitors (Kegge and Pierik 2010). Allelochemicals can be root exudates or BVOCs that have negative effects on competitors. Aboveground prospective or actual limitation of light through shading by neighbor plants can also induce competition responses, such as shade avoidance or shade acclimation (Vandenbussche, Pierik, Millenaar, Voeselek, & Van Der Straeten, 2005). The latter results in leaf area expansion and reduction of the chlorophyll a:b ratio to maximize light harvesting under shade conditions. In contrast shade avoidance includes stem and petiole elongation, as well as upward bending of leaves and early flowering, to reach over neighbors. Which competition strategy plants use depends on the species. For instance, the tobacco plant *Nicotiana tabacum* responds with shade avoidance (Ronald Pierik, Whitelam, Voeselek, de Kroon, & Visser, 2004).

Light quality and quantity in dense plant stands are extremely different to direct sunlight. In dense stands red (655 –665 nm) and blue light (400 –500 nm) are absorbed to provide energy for plant photosynthesis. In contrast far-red light (700 –800 nm) is transmitted. Thus, amounts of blue, red (R) and far red (FR) light depend on plant density (Franklin, 2008). Whereas a decrease in the red:far-red ratio (R/FR) occurs before canopy closure, since plants already receive transmitted FR light from neighbors, depletion of blue light does not occur until plants are shaded (Ronald Pierik, Cuppens, Voeselek, & Visser, 2004). Thus, a decrease in the R/FR ratio is the first light-mediated sign of upcoming shade. R and FR is detected by phytochrome photoreceptors, which detect the R/FR ratio (reviewed in Ronald Pierik and de Wit 2014). While R activates phytochromes (Pfr), FR inactivates them (Pr). Thus, the Pr/Pfr ratio reflects the R/FR. The activated phytochromes migrate from the cytosol into the nucleus and inhibit expression of several growth-promoting genes by interaction with, and degradation of different phytochrome interacting factors (PIF). In contrast at low R/FR most of the phytochromes are inactivate, and uninhibited PIF induce syntheses of several growth-promoting molecules (Chory & Wu, 2001). In addition to low R/FR, a decrease in blue light occurs as a result of canopy closure (Casal & Ballare, 2000). Blue light is perceived by two other classes of photoreceptors: cryptochromes and phototropins (Vandenbussche et al. 2005). While cryptochromes and phytochromes trigger direct vertical growth under low blue light in high plant densities, phototropins induce so-called "phototropic responses", wherein plants grow towards gaps in canopies with more light (Pedmale, Celaya, & Liscum, 2010).

In addition to light, BVOCs play an important role in neighbor detection and competition. Plants produce a range of different BVOCs which consist mainly of four biosynthetic classes: terpenoids, green leaf volatiles (GLVs), aromatic and non-aromatic amino acid derivatives (Kegge and Pierik 2010). They are emitted aboveground through leaves, stems or flowers or belowground by roots. Since plants are sessile organisms, BVOCs are very important for communication and interaction with their remote environment. Depending on the qualitative and quantitative composition of emitted blends, BVOCs have various advantages for the emitter, but also provide information about its physiology to other organisms. For instance they provide information about sexual receptivity, fruit maturity, insect damage, oviposition or competitive status (Baldwin, 2010). BVOCs play important roles in plant-insect interactions (Baldwin, 2010). For example plants recruit insects for pollination and seed dispensation, but also for their protection against herbivory by attracting herbivore predators (Clavijo McCormick, Unsicker, & Gershenson, 2012). Additionally, BVOCs play important roles in plant-plant interactions (Arimura, Shiojiri, & Karban, 2010). Some plants are able to eavesdrop on volatile blends released by plants in their direct vicinity, and adjust to them. For example when there are herbivore-infested plants in the neighborhood, undamaged plants may prime or induce defenses against herbivore attack. After wounding, specific blends of volatile compounds are emitted, that can be sensed by

surrounding plants. Furthermore, BVOCs can induce allelopathy and thus inhibit germination, growth and development of neighbor plants. Such volatiles are counted as allelochemicals and have direct or indirect effect on competitors. Particularly terpenoids and GLVs play an important role in interplant communication and allelopathy. Beside BVOCs and allelochemicals, phytohormones such as auxin, gibberellin, brassinosteroids and the volatile hormone ethylene are involved in plant-plant interactions (L. P. Tran & Pal, 2014). Especially ethylene (ET) plays an important role in shade avoidance. Under low R/FR conditions, ET biosynthesis and emission are elevated, which result in ET accumulation within canopies (Kegge & Pierik, 2010). Plants can sense exogenous environmental ethylene via a specific cell wall-located ethylene receptor, and respond with stem elongation and angled upward movement of leaves (R. Pierik, Visser, De Kroon, & Voeselek, 2003).

3.2 Green leaf volatiles

GLVs, including the aldehydes 1-hexanal, *cis*-3-hexenal and *trans*-2-hexenal, their corresponding alcohols 1-hexanol, *cis*-3-hexenol and *trans*-2-hexenol, as well as the *cis*-3-hexenyl and *trans*-2-hexenyl esters, are produced via the oxylipin pathway in plants. First membrane lipids including phospholipids and galactolipids are degraded by lipase to release linoleic and linolenic acids (Matsui, 2006). α -linoleic and α -linolenic acids are further converted by 13-LOX enzymes, which catalyze the stereospecific oxygenation of the 13th position to linoleic or linolenic acid 13-hydroperoxide (13HPOT). In *N. attenuata* two different 13-LOXes, LOX2 and LOX3, channel α -linolenic 13-HPOT into two distinct branches of the oxylipin pathway (Allmann, Halitschke, Schuurink, & Baldwin, 2010). Whereas the LOX3 branch results in biosynthesis of jasmonic acid (JA), the 13-HPOT provided by LOX2, is finally converted by hydroperoxid lyase (HPL) to *cis*-3-hexenal and its derivatives.

GLVs are important for plants to interact with several organisms in their environment. They can have effects on microorganisms, insects and also other plants (Matsui, 2006). Healthy and intact plants release GLVs in very low amounts, whereas after tissue disruption GLVs are rapidly formed and emitted (Matsui, 2006). Inducers include biotic stresses like pathogens or herbivory, as well as abiotic stresses like wind, drought or frost. Together with other BVOCs they provide a specific blend dependent on the kind of disruption. Neighbor plants can eavesdrop on the emitted volatiles and even seem to discriminate among different blends. In consequence neighboring plants develop various responses, presumably in preparation for attack. To date it is unclear how GLVs can be perceived. However, there are speculations that GLVs can be trapped and concentrated on the leaf tissue (Matsui, 2006). For emitter plants GLVs can function as indirect defense responses by attracting predators of the plants' attackers (A. Kessler &

Baldwin, 2001). In addition, GLVs can serve in direct defense by having a toxic effect on microbes, fungi and insects (Halitschke, Keßler, Kahl, Lorenz, & Baldwin, 2000). However, GLVs can also help these organisms to find potential host plants (Matsui, 2006).

3.3 Ethylene

ET is synthesized in two steps from S-adenosyl methionine (SAM). At first, SAM is converted to aminocyclopropanecarboxylate (ACC) by aminocyclopropanecarboxylate synthetase (ACCS). In the second step, ACC is oxidized by ACC oxidase (ACO) to ET (Johnson and Ecker 1998).

The production of ET is triggered in many ways including wounding, pathogen attack, flooding, shade avoidance, fruit ripening, development, senescence, and ethylene treatment itself (Johnson & Ecker, 1998). For ET there is a close connection between production and perception, since it is perceived by membrane-located ET receptors which are negative regulators of ET production (Baldwin, 2010). Like GLVs, ET is also released after wounding, herbivore or pathogen attack and increases either susceptibility or resistance, depending on the plant and infestation (Johnson & Ecker, 1998). In flooding situations some plants are able to develop so-called submerge avoidance. When plants are flooded, ET accumulates in stems as a first signal of flooding conditions, which leads to stem growth to escape the water (Ronald Pierik et al., 2007). Furthermore, as mentioned above, ET is an important inducer of the shade avoidance response. In dense canopies plants elevate ET emissions in response to low R/FR, which leads to high ET concentrations and thus stimulates plants to increase stem length and leaf angles (Ronald Pierik & de Wit, 2013). Additionally, the phytohormone ET is involved in the germination, emergence, and growth of seedlings, as well as fruit ripening and senescence in later stages (Johnson & Ecker, 1998).

3.4 Experimental overview

All things considered, ET and GLVs are involved in many plant processes and interactions with other organisms. Both are important for plants to successfully compete with others and increase fitness. Whereas GLVs have a great role in plant defense and thus protection and survival, ET is predominantly involved in growth and developmental processes as well as reproduction and fine-tuning of defense responses. To date it is unclear whether GLVs and ET interact with each other to synergistically increase plant competitive success. A previous study by Ruther and Kleier (2005) demonstrated that ET increases volatile emission from *Zea mays* plants stimulated by exposure to *cis*-3-hexenol. Thus, we hypothesize that ET and GLVs could have synergistic effects in competition.



Figure 2: *N. attenuata* plant in its natural habitat, the desert of Utah. Picture was taken by Danny Kessler.

To test this hypothesis, we constructed test populations of seedlings manipulated in ET and GLV biosynthesis, and measured growth parameters. *Nicotiana attenuata* Torrey ex Watson (Figure 1) seedlings were used as a model organism. *N. attenuata* is a species of wild tobacco that grows naturally in large monocultures after wildfires in the Great Basin Desert of southwestern Utah in the USA.

In the experiments different transformed *N. attenuata* genotypes were used, which were silenced in expression either of the ethylene biosynthetic gene *aminocyclopropane-carboxylate oxidase* (*NaACO*) or the GLV biosynthetic gene *lipxygenase 2* (*NaLOX2*), as well as a cross of both genotypes in which both genes were silenced by inverted repeat (ir) expression of a portion of the target gene, leading

to RNA interference and degradation of target transcripts (von Dahl et al., 2007; Allmann et al. 2010; Hannon 2002). In addition the empty vector line (EV) served as a transformation control.

In our experiments we observed the aforementioned *N. attenuata* genotypes in various seedling populations (Figure 3). These populations either contained the *N. attenuata* genotypes in monoculture or in 1:1 mixed cultures. Furthermore external GLVs were added to some monocultures.



Figure 3: *N. attenuata* seedling population in its natural habitat, the desert of Utah. Picture was taken by Danny Kessler.

The aim of our investigations was to observe the population-level effects of GLV and ET production in competing seedlings. Based on previous discoveries in plant-plant interactions, we hypothesized that ET emission and GLV production by neighbor plants synergistically increase competing seedlings' photosynthetic rates and growth rates. Furthermore, we asked whether this response depends on seedlings' own ability to produce GLVs and ET. Parallel to these questions we wanted to find out how GLV supplementation influences these effects. Our study demonstrates that seedlings are not strongly influenced by ET or GLVs from neighbors, but by their own production, and by external GLV supplementation.

4. Material and Methods

4.1 Chemicals

All chemicals were acquired from Sigma-Aldrich (St. Louis, USA), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or VWR (Darmstadt, Germany).

4.2 Germination media

Germination media was prepared according to Gamborg, Miller, and Ojima (1968). We dissolved 3.16 g Gamborg's B5 containing different salts and vitamins in a 1 L bottle with 980 mL distilled water and a stir bar. Subsequently the pH was adjusted to 6.8 with sodium hydroxide solution and 6 g phytigel was added. During the whole procedure the bottle stood on a magnetic mixer (IKA, Staufen, Germany) to constantly stir the medium and prevent agglutination. After preparation, medium was autoclaved (H+P Labortechnik GmbH, Oberschleißheim, Germany) at 121 °C for 20 min and then cooled in a 65 °C water bath. The medium was poured into 100x20 mm (diameter x height) petri dishes under a sterile bench (Kendro, Newtown, USA) and left to solidify. Petri dishes with media were stored in a refrigerator (Liebherr, Ochsenhausen, Germany) at 4 °C until they were used for germination.

4.3 Plant material and germination

Nicotiana attenuata grows naturally in large monocultures after wildfires in the Great Basin Desert of southwestern Utah in the USA. For our investigations, transgenic *N. attenuata* plants, which either had the *aminocyclopropanecarboxylate oxidase* (*NaACO*) gene (irACO; line A-03-321-10-1), the *lipxygenase 2* (*NaLOX2*) gene (irLOX2; line A-03-321-10-1), or both genes silenced (irACOirLOX2) in the WT genetic background were used. A line containing the empty pSOL3 vector (EV, line A04-266-3-2) was used as a control. Plant transformation was conducted with an inbred line of the UT genotype, as previously described (Krügel, Lim, Gase, Halitschke, & Baldwin, 2002).

Prior to germination, seeds were first sterilized for 5 min with dichloroisocyanuric acid, supplemented with 50 µL 0.5 % Tween-20 detergent (Merck, Darmstadt, Germany) and rinsed three times with sterilized distilled water (Krügel et al., 2002). Subsequently seeds were incubated for 1 h in 5 mL of 50 x diluted liquid smoke (House of Herbs,

Inc.: Passaic, New Jersey, USA) supplemented with 50 μL of 0.1 M gibberellic acid (GA_3) to stimulate germination. Afterwards, seeds were rinsed three times with sterilized distilled water and transferred to germination medium. All steps were conducted inside a laminar flow hood (Kendro, Newtown, USA). After treatment seeds were transferred to the 100x20 mm petri dishes with germination medium. Lids were closed and sealed around with parafilm. Petri dishes were stored in a Percival chamber (Percival Scientific, Perry, USA) at 26°C and 100% light for about 13 to 15 days.

4.4 Experimental set-up

All experiments were performed with *N. attenuata* plants in seedling stage which were prior germinated and cultivated as previous described in “Plant material and germination”. Seedlings with the most uniform growth were randomly assigned to appropriate population types. At four-leaf-stage, after 13- 15 days, seedlings were transferred to transparent 1.4 L polypropylene boxes (LOCK&LOCK, Frankfurt/Main, Germany), filled with 0.2 L clay granulate (Fibo ExClay, Lamstedt, Germany) on the bottom and 0.7 L standard *N. attenuata* potting (Klasmann, Geeste, Germany) (Figure 3).

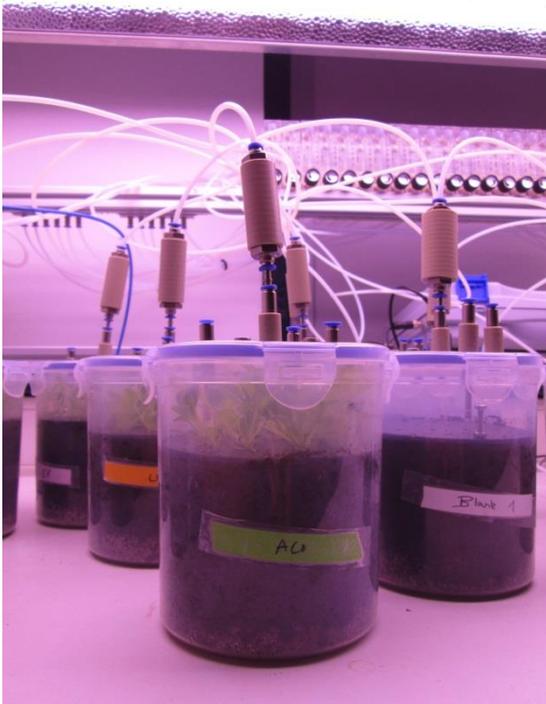


Figure 3: Experimental set-up maintained in a darkened laboratory under fluorescent lamps. Transparent polypropylene boxes contained seedling monocultures, combined seedling populations or planting substrate without seedlings (blank boxes). Boxes had a constant air in- and outflow of 0.1 L min^{-2} set through flow meters.

Boxes were covered with airtight transparent lids containing several lockable holes (Figure 4). Two holes were needed for a constant air in- and outflow of 0.1 L min^{-2} . The remaining holes were closed with steel pins.



Figure 4: Box viewed from the top: the airtight lid is equipped with connections for air in- and outflow. Ambient air from the experimental environment was cleaned with an active carbon filter and pushed into individual seedling boxes with a compressed-air pump.

Ambient air from the experimental environment, cleaned with an active carbon filter, was pushed into each of the individual seedling boxes with a compressed-air pump (model DAA-V114-GB; Gast Mfg., Benton Harbor, Mich.). Per box, one flow meter was used to regulate the airflow (Key instruments, Trevose, USA). Planting substrate was covered with a black 4 mm thick polypropylene plastic plate (Kyma Technologies, USA) with sixteen 2 mm holes to separate the shoot compartment from the substrate. Seedlings were planted carefully through the holes. They were positioned in square grids to reach a density of ca. 3555 plants m^{-2} . The boxes were maintained in a darkened laboratory under “fluora” fluorescent lamps (OSRAM, München, Germany) with 16 h light ($160 \mu\text{m s}^{-1} \text{m}^{-2}$) and 8 h dark. There was also one box with a sensor for temperature and humidity measurements.

For the experiment seedlings were cultivated as previously described in “Plant material and cultivation”. Boxes were filled with different *N. attenuata* seedling populations, which consisted of various genotypes in monoculture or 1:1 mix (Table 1). Additionally, blank boxes without seedlings were included for background measurements. In total there were two rounds of the experiment under the same conditions. For the first experiment, we had one replicate of each genotype combination and monoculture to test the experimental conditions and methods. The second round was conducted with five replicates per population type, except the irACO populations. Due to germination problems there was only one replicate per irACO population. In this experiment some monocultures were additionally supplemented with GLVs. For both experiments gas exchange and leaf area was measured in all boxes. When seedlings had maximum size, ET production of all populations was measured. Finally seedlings were harvested to measure shoot biomass, internal GLVs and silencing efficiency.

Table 1: Monocultures and genotype combinations with or without GLV supplementation used in experimental set-up.

Population	Population type	GLVs		ET
		Internal	External	
EV	Monoculture	+	-	+
irLOX2		-	-	+
EV + GLVs		+	+	+
irLOX2 + GLVs		-	+	+
irACO		+	-	-
irLOX2xirACO		-	-	-
EV/irACO	Mixed culture	+	-	+/-
EV/irLOX2		+/-	-	+
EV/irLOX2xirACO		+/-	-	+/-
irLOX2xirACO/irACO		+/-	-	-
irLOX2xirACO/irLOX2		-	-	+/-

4.5 GLV treatment

For GLV treatment, a mixture of GLVs in lanolin paste was prepared. Therefore, two 50 mL tubes, filled with 50 mL lanolin each, were heated to 50 °C in a water bath (Memmert GmbH, Schwabach, Germany). When lanolin was liquefied 15.64 µL of GLV mix were pipetted to one tube and the lanolin was mixed well. GLV mix was prepared as described in Table 2. Subsequently both lanolin paste and GLV-lanolin paste were separately filled in 1 mL syringes and stored at -20 °C in a freezer (Siemens AG, München, Germany). For GLV supplementation, boxes were equipped nightly at 9 p.m. either with 1 mL lanolin paste enriched with GLVs (+GLV) or with 0.1 mL lanolin without GLVs (-GLV) as control (Table 2).

Table 2: Protocol for preparation of GLV mix.

Compound	Volume [μL]
<i>Cis</i> -3-hexenal	722.40
<i>Trans</i> -2-hexenal	318.34
<i>Cis</i> -3-hexen-1-ol	208.99
<i>Trans</i> -2-hexen-1-ol	287.94
<i>Cis</i> -3-hexenyl-acetate	5.19
<i>Trans</i> -2-hexenyl-acetate	3.95
<i>Cis</i> -3-hexenyl-propanoate	1.01
<i>Trans</i> -2-hexenyl-propanoate	0.90
<i>Cis</i> -3-hexenyl-butanoate	10.96
<i>Trans</i> -2-hexenyl-butanoate	3.99

4.6 Determination of leaf area

Leaf area expansion (LAE) of individual seedlings in all populations was determined over a certain time frame. Every morning top view pictures of all boxes were taken with a web cam. Leaf area of individual seedlings was calculated with ImageJ, a public domain Java image processing program (Abràmoff, Magalhães, & Abràmoff, 2004). When seedlings started to overgrow each other, individual seedlings could not be distinguished anymore. From this time point on, area of whole populations was determined and normalized to the count of plants in this population to calculate a value for leaf area per seedling.

4.7 Gas exchange measurements

Gas exchange measurements (CO_2 and H_2O) were taken with an open-system portable infrared gas analyzer (IRGA; Li-Cor 6400, Li-Cor Inc., Lincoln, NE, USA) equipped with a customized leaf chamber and a CO_2 injection system (Figure 5; model 6400-01, Li-Cor Inc., Lincoln, NE, USA) adjusted to a constant incoming CO_2 concentration of $400 \mu\text{mol CO}_2 \text{ mol air}^{-1}$. The customized leaf chamber could be placed directly on top of boxes.

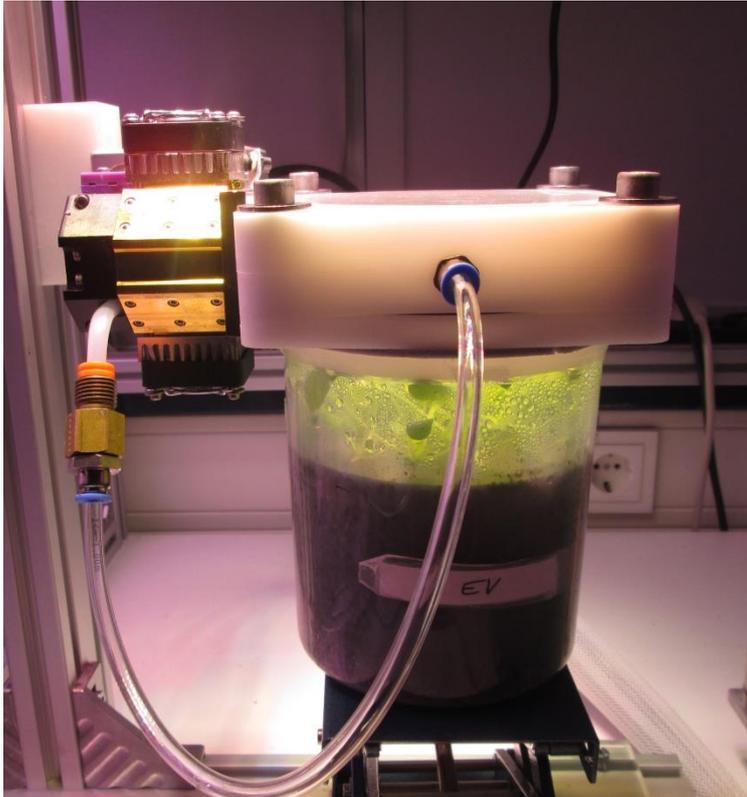


Figure 5: EV monoculture box during gas exchange measurement with customized leaf chamber on top.

With our set-up, CO₂ concentration and humidity were measured to determine photosynthetic rate and transpiration rate of each seedling populations. Calculation was based on difference of CO₂ and H₂O concentration between sample cuvette and reference cuvette (Figure 6). In the sample cuvette, the air stream from the headspace of boxes was measured, whereas the reference cuvette represented the air stream that was piped into the boxes. The detection was conducted with infra-red gas exchange analysis (IRGA).

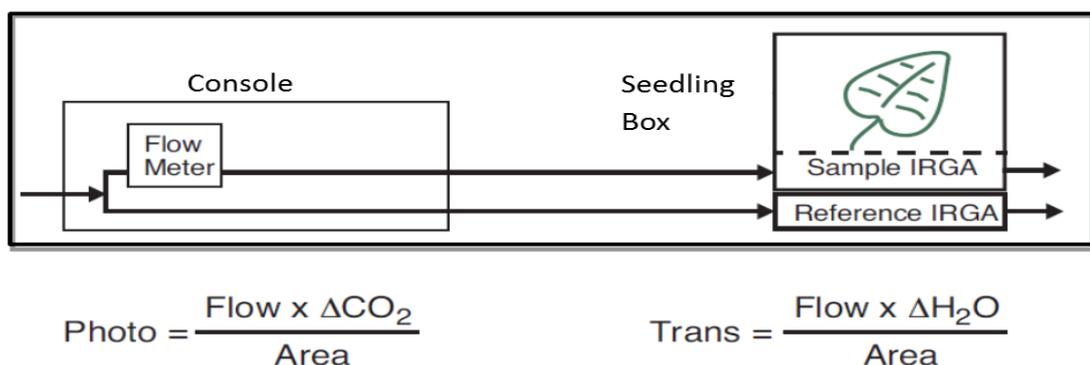


Figure 6: Structure and operating principle of LI-COR 6400 and its calculation of photosynthesis (Photo) and transpiration (Trans) in response to ΔCO_2 and $\Delta\text{H}_2\text{O}$. Delta CO₂ and delta H₂O were determined from the difference of the CO₂ and H₂O concentrations between sample cuvette and reference cuvette. Concentrations were measured with infrared gas analyzers (IRGAs).

The light intensity for all gas exchange measurements was 1100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by a custom-built external LED light source (Figure 21; materials from Roschwege,

Greifenstein, Germany, constructed by D. Veit). After placing the chamber on top of the box, it took about 5 to 10 minutes for the CO₂ concentration to stabilize. The CO₂ and H₂O concentration was subsequently measured for 3 minutes. To determine the background of the CO₂ produced by the planting substrate and the incoming air, the control boxes without seedlings were measured in the same way.

4.8 ET measurement of whole seedling populations

ET production of different seedling populations was measured at the end of the experiment, shortly before harvest, when seedlings were largest. Emissions were determined continuously and non-invasively in real-time with a photo-acoustic trace gas sensor (ETD-30, Sensor Sense, Nijmegen, NL), which was able to detect on-line 300 parts per trillion volume of ethylene within 5 s (Cristescu, Persijn, te Lintel Hekkert, & Harren, 2008). It consisted of a CO₂ laser and a photoacoustic cell, in which the gas was detected. The laser radiation inside the photoacoustic cell could be absorbed by ET and released as heat, which created a pressure increase inside a closed volume. A periodic pressure wave was generated by changing the laser at an acoustic frequency. The pressure wave could be detected with a sensitive miniature microphone. Finally, the ethylene concentration was calculated from comparison of the photoacoustic signals on various laser lines and absolute ET concentrations were calculated based on prior calibration of the laser with a reference ethylene gas.

To measure the headspace of the whole populations inside boxes, closed boxes were connected with the air in- and outflow of the instrument, which was regulated by an automated valve control box (VC-6, Sensor Sense, Nijmegen, NL). Boxes were continuously flushed with a flow of air passed through activated charcoal and a catalyzer (Sensor Sense, Nijmegen, NL) at 200 °C, to oxidize all organics, at 5 L h⁻¹. The air from samples was additionally conducted through a KOH and CaCl₂ scrubbers, to remove CO₂ and H₂O. To eliminate the background, which came mainly from soil, ET concentrations measured for blank boxes were subtracted. In the end, results for all populations were normalized to the harvested seedling biomass.

4.9 ET measurement of different seedling genotypes

In addition to ET production of whole seedling populations inside the boxes we determined ET production of individual *N. attenuata* seedlings of different genotypes. Seedlings were reared under the same condition as previous described in “*N. attenuata* cultivation” and transferred 10 to 14 days after germination to experimental set-up. All boxes were equipped with four seedlings per genotype, for a total of 16 seedlings. After

14 days of adaption, ET production was measured. Prior to measurements, the seedlings of three boxes were carefully harvested from boxes and weighed to determine biomass. Afterwards, they were placed into airtight 4 mL vials with polytetrafluorethylen (PTFE) septum lids (Sigma Aldrich, St. Louis, USA). Lids were additionally sealed around the rim with PTFE tape. Vials were laid on their side and incubated for 4 h in a Percival chamber (Percival Scientific, Perry, USA) at 26°C and 100% light to accumulate ET. Emissions were determined in stop-flow mode with a photo-acoustic trace gas sensor (ETD-30, Sensor Sense, Nijmegen, NL) as previous described in “ET measurement of whole seedling populations”, but vials were flushed with a flow of purified air at 2 L h⁻¹.

4.10 Collection of GLVs

Volatiles were trapped from headspaces of the GLV-supplemented EV and irLOX2 boxes to determine headspace GLV concentrations. Additionally, the headspace of blank boxes without seedlings or GLV addition was measured as a control. During the collection boxes were closed. Collection was performed as described by Halitschke (2000). Volatiles were trapped on 20 mg of a polymeric adsorbent (SuperQ; Alltech, Deerfield, Ill.), secured with glass wool in small glass cylinders (ARS, Inc., Gainesville, FL, USA). For the volatile collection, traps were connected to the outflow of the boxes. The boxes had the normal constant air inflow of 0.1 L min⁻¹ ambient air purified with an activated charcoal filter. To generate a constant air outflow of 0.1 L min⁻¹ through the filters, air was flushed with a vacuum pump (model DAA-V114-GB; Gast Mfg., Benton Harbor, Mich.) connected to the filters and regulated with mass flow meters (Key instruments, Treviso, USA). Thus, filters were continuously perfused with the seedling population headspace air. The pumps were connected to a vacuum manifold, which allowed volatiles to be collected from up to 53 chambers simultaneously. After 24 h collection, each filter was spiked with 320 ng of tetralin internal standard (IS) in hexane (Sigma-Aldrich) and eluted with 250 µL of dichloromethane into a 1.5 mL glass screw-cap GC vial containing a 250 µL microinsert. Afterwards, samples were analyzed by GC-QMS (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan).

4.11 Measurement of internal GLVs

Internal GLVs of different *N. attenuata* genotypes reared in distinct populations were measured at the end of the experiment from the harvested seedlings. Shoots of all seedlings were harvested, weighed and frozen in liquid nitrogen. Frozen tissues were ground with a pestle in a mortar (Haldenwanger, Waldkraiburg, Germany) over liquid nitrogen. Afterwards, 50 mg of tissue was aliquoted into 1.5 mL tubes. All samples

were removed from nitrogen, and 800 μL saturated CaCl_2 solution was immediately added to inhibit enzyme activity. This solution was supplemented with 5 $\mu\text{L}/100\text{ mL}$ *cis*-4-hexenol internal standard. The solution was previously prepared by dissolving 96.667 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ in 100 mL Millipore water. To produce 1 $\mu\text{g}/\mu\text{L}$ methanol of the internal standard *cis*-4-hexenol (d^{20}_4 : 0.857) we added 1.17 μL *cis*-4-hexenol to 1 mL methanol. Finally, 5 μL of the standard mixture were pipetted into 100 mL CaCl_2 solution. After adding the solution one 25 mm long piece of polydimethylsiloxane (PDMS) tubing (1 mm i.d. x 1.8 mm o.d., Carl Roth) was added to each sample and incubated overnight in a desktop shaking incubator (Eppendorf, Hamburg, Germany) at 25 °C and 800 rpm. PDMS pieces were gently removed with a tweezers and transferred to 1.5 mL GC vials with 1 mL distilled water each. To detach most tissue from the tubing, vials were shaken on a vortex mixer (Scientific Industries, New York, USA). Subsequently, PDMS was removed with a tweezers and dried under a gentle stream of nitrogen. For the following analysis of internal GLVs by TD-GC-MS, each PDMS piece was placed in an 89 mm glass TD tube (Supelco-Sigma-Aldrich, St. Louis, USA) and put on the TD-20. After qualitative and quantitative analyses of the samples, internal GLVs of each seedling were normalized to extracted seedling shoot biomass.

4.12 Preparation of PDMS

Prior to volatile collection, small PDMS pieces were prepared from silicon tubing (ST) with an inner diameter of 1 mm and an outer diameter of 1.8 mm (Roth, Karlsruhe, Germany). They were cut with a razor blade and a handmade cutting mask into 25 mm pieces. Afterwards, PDMS pieces were soaked for at least 3 h in a 4:1 mixture of acetonitrile/methanol and then solvent was decanted. At the end they were baked at 210 °C for 5-6 h in a modified heating oven under constant N_2 gas flow of 5 L min^{-1} . Within 1.5 h the oven temperature was ramped to 210 °C and held for 3.5-4.5 h. The cleaned and baked volatile-free PDMS pieces were kept in brown glass vials under argon to prevent contamination during storage.

4.13 GC-QMS analysis

The qualitative and quantitative analysis of the GLV-supplemented box headspaces and internal leaf GLVs was conducted with a TD-20 thermal desorption unit (Shimadzu, Kyoto, Japan) connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu, Kyoto, Japan) as previously described by Kallenbach et al. (2014). For the internal GLV analysis each PDMS piece was positioned in one 89 mm glass TD tube (Supelco-Sigma-Aldrich, St. Louis, USA) and placed on the TD-20. At first volatiles were

desorbed from PDMS under a nitrogen stream at 100 mL min⁻¹ for 15 min at 200 °C and cryo-focused at -20 °C onto a Tenax® adsorbent trap. The adsorbent trap was in front of the Rt-Wax column (30 m long, 0.25 mm i.d., 0.25 film thickness; Resteck). Subsequently the trap was heated within 10 s to a temperature of 230 °C. All adsorbed analytes were injected with a split ratio of 1 to 20 onto the column. Helium was used as carrier gas at a constant linear velocity of 40 cm s⁻¹.

For analysis of the GLV headspace samples we used the same instrument as described for internal GLV PDMS samples, except that a liquid autosampler (AOC-20i, Shimadzu) injected 1 µL of sample, splitless, into the GC inlet held at 230 °C and a Rtx-5 MS column (30 m long, 0.25 mm i.d., 0.25 film thickness; Resteck) was used.

For internal GLVs, the GC oven was held at 40 °C for 5 min, then ramped to 115 °C at 5.0 °C min⁻¹, and finally to 230 °C at 30 °C min⁻¹, where it was held for 0.17 min, and for GLV headspace samples the GC oven was held at 40 °C for 5 min, then ramped to 185 °C at 5.0 °C min⁻¹, and finally to 280 °C at 30 °C min⁻¹, where it was held for 0.83 min. The electron impact (EI) spectra was recorded at 70 eV in scan mode from 33 to 400 m/z using a scan speed of 2,000 Da s⁻¹. The transfer line was held at 240 °C and the ion source at 220 °C. Data processing against our reference compounds (Table 3, 4) was performed using the Shimadzu GCMS solutions software (<http://www.shimadzu.com/an/gc/advflowtech/sw-dl.html>). Compounds in Table 3 and 4 were identified by comparison of spectra and Kovats retention indices to libraries or by comparison to pure standards. For the tetralin-spiked GLV headspace samples, quantified peaks were finally normalized to the elution internal standard, tetralin, and indicated as percentage tetralin peak area (A. M. Kallenbach et al., 2014).

Table 3: List of 8 compounds used for automated peak identification and integration of chromatograms for internal GLV analysis (RT: retention time).

No.	Class	Compound	RT [min]	Target ion [m/z]
1	Green leaf volatiles	<i>cis</i> -3-hexen-1-al	7.56	41
2		<i>trans</i> -2-hexen-1-al	9.84	55
3		<i>cis</i> -3-hexen-1-ol	15.04	41
4		<i>trans</i> -2-hexen-1-ol	15.46	57
5		hexan-1-ol	14.86	56
6		<i>cis</i> -3-hexen-1-ol-acetate	12.87	67
7		<i>cis</i> -3-hexenyl isobutanoate	15.15	43
8		<i>cis</i> -3-hexenyl butanoate	17.12	67

Table 4: List of 13 compounds used for automated peak identification and integration of chromatograms for GLV headspace analysis (RT: retention time).

No.	Class	Compound	RT [min]	Target ion [m/z]
1	Green leaf volatiles	<i>cis</i> -3-hexen-1-al	5.65	69
2		<i>trans</i> -2-hexen-1-al	7.67	83
3		<i>cis</i> -3-hexen-1-ol	7.84	67
4		<i>trans</i> -2-hexen-1-ol	8.23	57
5		hexan-1-ol	8.30	56
6		<i>cis</i> -3-hexen-1-ol-acetate	13.33	67
7		<i>cis</i> -3-hexenyl isobutanoate	17.76	67
8		<i>cis</i> -3-hexenyl butanoate	19.07	67
9		<i>cis</i> -3-hexenyl-2-me-butanoate	20.42	82
10		<i>cis</i> -3-hexenyl valerate	20.52	67
11		<i>cis</i> -3-hexenyl <i>trans</i> -2-me-butenoate	23.01	82
12		<i>cis</i> -3-hexenyl caproate	23.42	82
13	Other	Tetralin	18.72	194

4.14 Determination of shoot biomass

Shoot biomass of all seedlings was measured at the end of the experiment after 13 days. Shoots were cut from roots one by one and weighed on a scale (Sartorius AG, Göttingen, Germany) finally.

4.15 Silencing efficiency test

4.15.1 Tissue grinding

At the end of the experiment, shoots of all seedlings were harvested, weighed and flash-frozen in liquid nitrogen. Frozen tissues were ground with a pestle in a mortar (Haldenwanger, Waldkraiburg, Germany). Finally, 100 mg of all samples were aliquoted in 1.5 mL tubes for small-scale RNA isolation.

4.15.2 Small scale RNA isolation

RNA isolation samples were kept at -80 °C or on liquid nitrogen until extraction. Upon removal from liquid nitrogen, 1 mL TRIzol© (Life Technologies GmbH, Darmstadt, Germany) was added per sample. Afterwards, tubes were shaken at RT for 5 min (Vortex Genie, Scientific Industries, New York, USA) and finally centrifuged

(Eppendorf, Hamburg, Germany) for 10 min at 4 °C and 12000 g. Supernatants were transferred to a new set of 1.5 mL tubes and pellets were discarded. Subsequently supernatants were dosed with 0.2 µL Chloroform, shaken for 15 s and kept at RT for 2-3 min. Samples were centrifuged for 10 min at 4 °C and 10000 g afterwards. We then collected the upper water phase which contained the RNA, transferred it to new 1.5 mL tubes and added isopropanol at 80 % of the volume of the collected water. Afterwards, samples were kept for 1 hour at RT to let the RNA precipitate and subsequently centrifuged for 10 min at 4 °C and 10000 g. As a next step the supernatants were removed and discarded. The leftover white pellets on the bottom of the tubes were washed with 75 % ethanol two times. To wash the pellet 75 % ethanol was pipetted to the sample, vortexed to move pellets through the ethanol and left standing at RT for 1-2 min. Afterwards all samples were centrifuged for 10 min at 4 °C and 7500 g and the ethanol was removed and discarded. Finally pellets were dried for a few minutes on air until they were white, and dissolved in diethylpyrocarbonate (DEPC)-treated water. DEPC destroys enzymatic activity by modifying -NH, -SH and -OH groups in RNases and other proteins. DEPC water was prepared by incubating shaking overnight at 37 °C with 0.1% diethylpyrocarbonate (DEPC) and then autoclaving twice to degrade the DEPC.

4.15.3 RNA Gel Electrophoresis

To check RNA quality we performed a RNA gel electrophoresis. A 1 % agarose gel was prepared without ethidium bromide. For the gel 1.4 g agarose was dissolved in 140 mL 1 x TAE buffer (Table 5). To obtain 1x TAE buffer, 50x TAE buffer was diluted 1:50 in water. Finally the solution was heated for 2 min in a microwave to melt the agarose (Siemens AG, München, Germany) and poured into a mold.

Table 5: Protocol for 50x TAE buffer.

<i>Reagent</i>	50x TAE buffer
Tris base	242 g
acetic acid	57.1 ml
EDTA (0.5 mM)	100 ml

RNA samples were vortexed and spun down. Afterwards, 1 µL of each sample was aliquoted into a fresh and clean microcentrifuge tube. Additionally, 2 µL 1 x RNA loading dye (Thermo scientific, Waltham, USA) were added and spun down. Subsequently RNA was denatured for 5-10 min at 65 °C and immediately moved on ice. Lastly denatured RNA was loaded on gel, run for 15-20 min at 100 V in a gel electrophoresis system (Bio-Rad Laboratories GmbH, München, Germany), and afterwards incubated for 15 min in 1x TAE buffer with 0.01 µl/mL ethidium bromide

(Sigma, St. Louis, USA) on a shaker (Infors AG, Bottmingen, Switzerland). At the end RNA was visualized with a bio imaging system (BioDoc-It, VWR, Radnor, Wales).

4.15.4 cDNA Synthesis

At first RNA concentrations were quantified on an UV-Vis-Spectrometer (NanoDrop, Arlington, USA). Based on that, 5 μ L RNA was diluted with DEPC water in 1.5 mL tubes to 0.5 μ g/ μ L (Eppendorf, Hamburg, Deutschland). Diluted samples were then vortexed and droplets were spun down in a centrifuge. Following the denaturation and reaction master mix was prepared (Table 6):

Table 6: Protocol for Denaturation and Reaction mix

DenaturationMIX:		Reaction MIX:	
<i>Reagent</i>		<i>Reagent</i>	
dNTP	0.5 μ L	5xRT Buffer	2 μ L
Oligo dT	0.5 μ l	RiboLocker	0.25 μ l
DEPC H ₂ O	1.5 μ l	RevertAid	0.075 μ l
		DEPC H ₂ O	2.75 μ l

For the reaction 2.5 μ L of diluted RNA and 2.5 μ L denaturation master mix was pipetted into a 96-well plate. The plate was kept on ice the whole time. Everything was spun down in a centrifuge (Eppendorf, Hamburg, Deutschland). The plate was covered with a SealMat (Eppendorf, Hamburg, Germany) and finally heated up at 65 °C for 5 min on a PCR machine (Thermo cycler, Eppendorf, Hamburg, Germany). Afterwards denatured RNA was chilled on ice for 1 min and 5 μ L of reaction master mix was pipetted into each well. Then the plate was spun down for 5 s, covered with a SealMat again and run on a PCR machine (Eppendorf, Hamburg, Germany) for 15 min at 70 °C. After the reaction was finished, 15 μ L of Millipore water were added to each sample.

4.15.4 PCR

Several cDNA samples were randomly taken to check sample quality by PCR. This method was used as a quality check, because the used *Taq* polymerase enzyme is cheaper, but standard PCR is at most semi-quantitative. At first the master mix was prepared on ice (Table 7):

Table 7: Protocol for master mix.

<i>Reagent</i>	Master Mix
10x buffer	5 μ L
MgCl ₂	4 μ L (25 μ M)
dNTP (10 mM)	1 μ L
Primer 1 (10 μ M)	1 μ l
Primer 2 (10 μ M)	1 μ l
Taq polymerase	1 μ L (1 U/ μ l)
MilliQ H ₂ O	36 μ l

The primers Nt_EF1a_FWD2 and Nt_EF1a_RVS2 were used to amplify a portion of the Elongation Factor 1a, a constitutively expressed gene which is also used as a standard to normalize the calculated transcript abundance of test genes in qPCR (Schuman, Palmer-Young, Schmidt, Gershenzon, & Baldwin, 2014). For the reaction 1 μ L of cDNA and 49 μ L master mix were pipetted into a 96-well plate. Immediately the plate was put into a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) and run under the following conditions (Table 8):

Table 8: PCR temperature program. Step 2, 3 and 4 were repeated a total of 35 times.

Step	Time [min]	Temperature [°C]
1	00:30	94
2	00:10	94
3	00:30	60
4	00:15	72
5	01:00	72
6	Hold	RT

4.15.4 DNA Gel-Electrophoresis

After PCR, products were assessed by DNA gel electrophoresis. DNA samples were vortexed and spun down. Afterwards 1 μ L of each sample was added to 2 μ L 1 x DNA loading dye (Thermo scientific, Waltham, USA) and pipetted into a fresh prepared 1 % agarose gel (Bio-Rad Laboratories GmbH, München, Germany) without ethidium bromide. The gel was run for 15-20 min at 100 V in a gel electrophoresis system (Bio-Rad Laboratories GmbH, München, Germany), and afterwards incubated for 15 min in 1x TAE buffer with 0.01 μ l/mL ethidium bromide (Sigma, St. Louis, USA) on a shaker (Infors AG, Bottmingen, Switzerland). At the end DNA was visualized with a bio imaging system (BioDoc-It, VWR, Radnor, Wales).

4.15.4 qPCR

After cDNA quality was shown to be sufficient, qPCR was performed using an Mx3005P qPCR system (Agilent Technologies, Santa Clara, CA, USA) to analyze silencing efficiency in irLOX2, irACO and irLOX2xirACO. qPCR was conducted with SYBR Green, an asymmetrical cyanine dye used as a nucleic acid stain to quantify double stranded DNA, and ROX. ROX is an inert dye, whose fluorescence does not change during the reaction, and thus it is used as passive reference. The SYBR analysis master mix was pipetted as follows (Table 9):

Table 9: Protocol for SYBR analysis mix.

<i>Reagent</i>	1x
10x buffer	2 μ L
MgCl ₂ (50 mM)	1.4 μ L
dNTP (5 mM)	0.8 μ L
Forward Primer (5 μ M)	1.2 μ l
Reverse Primer (5 μ M)	1.2 μ l
SYBR Green	0.6 μ l
ROX 10x diluted	0.8 μ l
MilliQ water	10.9 μ l
Hotgoldstar enzyme	0.1 μ l

For the reaction, 1 μ L of diluted RNA and 19 μ L of fresh prepared master mix were pipetted into a 96-well plate. In addition to Efla as a standard gene, reactions were conducted to amplify NaLOX2 (Allmann et al., 2010) and NaACO (von Dahl et al., 2007). The assembled plate was finally run on a qPCR instrument (Stratagene, California, USA) as follows (Table 10):

Table 10: Method used for qPCR. Step 2 and 3 were repeated a total of 40 times.

Steps	Time [min]	Temperature [°C]
1	10:00	95
2	00:15	95
3	1:00	60
4	00:15	95
5	00:30	55
6	00:15	95

The last three steps of the qPCR program generate a melting curve which was used to assess specificity.

5. Results

5.1 Screening of genotypes

To characterize all genotypes that were used, silencing efficiency, as well as ET emission and internal GLVs of individual seedlings were investigated. Seedlings of the genetically transformed inverted repeat lines irACO and irLOX2 were silenced in expression of *NaACO* or *NaLOX2*. The irLOX2xirACO line was a cross of both lines and thus silenced in *NaACO* and *NaLOX2*, whereas the EV line contained only the empty pSOL vector and had no silencing.

5.1.1 Silencing efficiency test

To check silencing efficiency of the transformed genotypes, gene expression of the *NaACO* gene (A) in irACO and irLOX2xirACO, as well as of the *NaLOX2* gene (B) in irLOX2 and irLOX2xirACO was compared to the respective expression in EV (Figure 5).

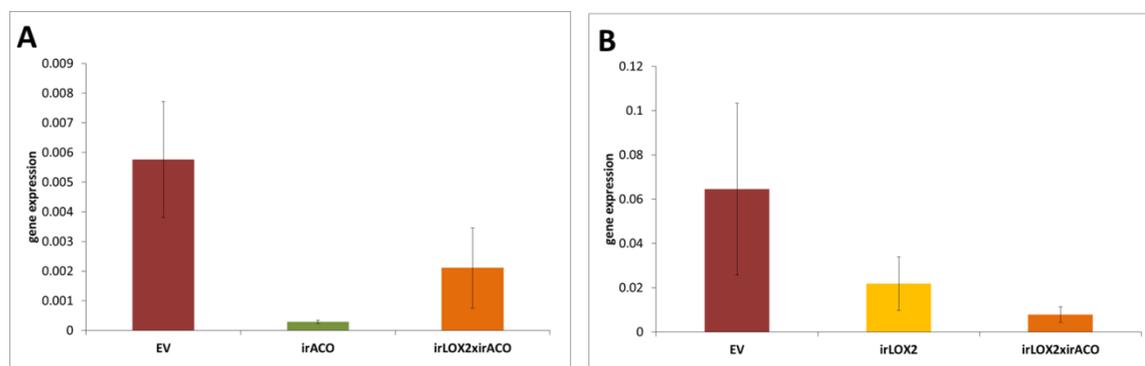


Figure 5: Silencing efficiency test of the *NaACO* (A) gene in irACO and irLOX2xirACO, as well as the *NaLOX2* (B) gene in irLOX2 and irLOX2xirACO compared to respective expressions in EV. Gene expression of *NaACO* (A) and *NaLOX2* (B) relative to expression of the *NaElf1* control gene was ascertained by qPCR.

The gene expression of *NaACO* was, due to inverted repeat silencing, in the irACO and irLOX2xirACO lines much lower than in the EV line (Figure 5A). For the expressions of *NaLOX2* the same effect was shown for the silenced genotypes. Thus, irLOX2 and irLOX2xirACO had reduced *NaLOX2* expressions by comparison with EV (Figure 1 B). Nevertheless there were also large deviations in measured expression of *NaACO* and *NaLOX2* between the respective silenced lines. Whereas irACO had an expression of *NaACO* of only 0.0003, irLOX2xirACO had one of 0.002. *NaLOX2* was expressed in irLOX2xirACO half times than in irLOX2. But despite high standard deviations, silencing was clearly demonstrated.

5.1.2 ET emission

The ET emission rates per shoot biomass were measured from individual seedlings of the respective genotypes, after seedlings were reared for 18 days in the experimental set-up (Figure 6).

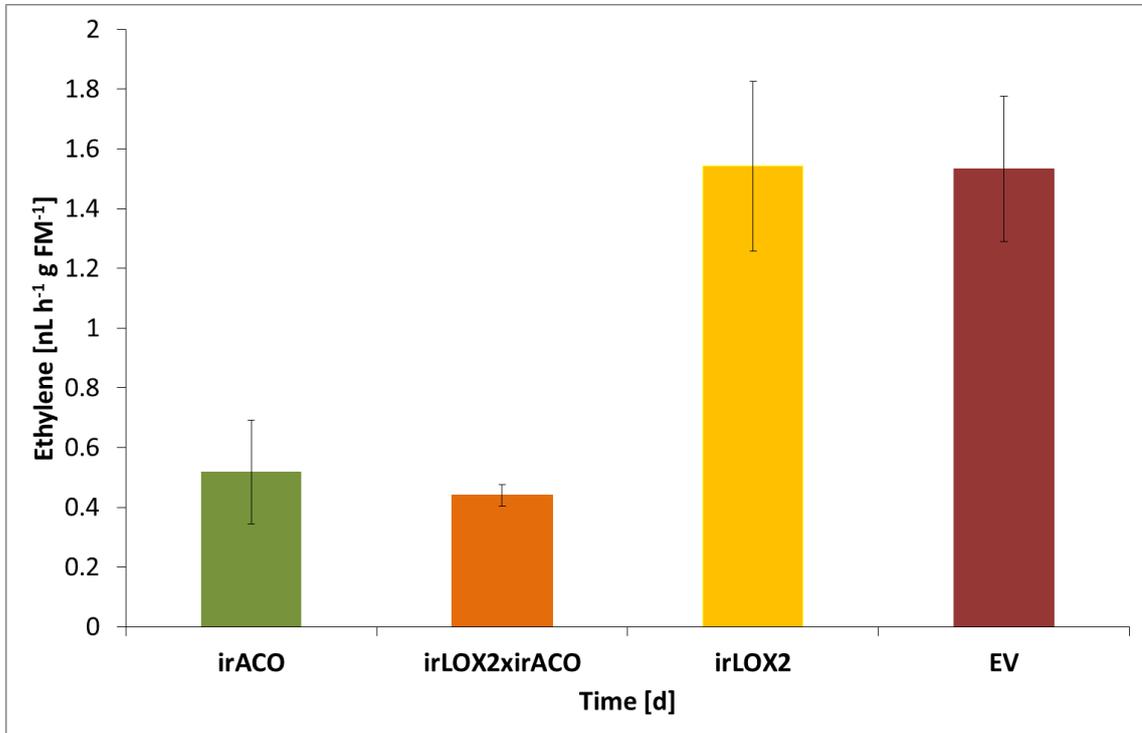


Figure 6: ET emission per shoot biomass of different *N. attenuata* seedling genotypes. Seedlings were harvested from boxes after 18 days and stored in 4 mL vials for 4 h to accumulate ET. Finally, ET concentrations were measured with a photo-acoustic trace gas sensor in stop and flow mode (n = 12 seedlings/genotype).

ET emission measurements of individual seedlings clearly demonstrated that genotypes silenced in *NaACO* (irACO, irLOX2xirACO) had ET emissions three times lower than EV and irLOX2 seedlings: seedlings of irACO and irLOX2xirACO had emission rates of about 0.5 nL h⁻¹ g FM⁻¹ while irLOX2 and EV seedlings emitted concentrations of about 1.6 nL h⁻¹ g FM⁻¹.

5.1.3 Internal GLV concentrations

Besides ET, also internal GLV concentrations per shoot biomass of the experimental genotypes were determined (Figure 7). Internal GLVs were isolated from seedlings of the monocultures in our main experiment (n = 5*). For the measurement seedlings were harvested 13 days after they were transferred to the experimental set-up.

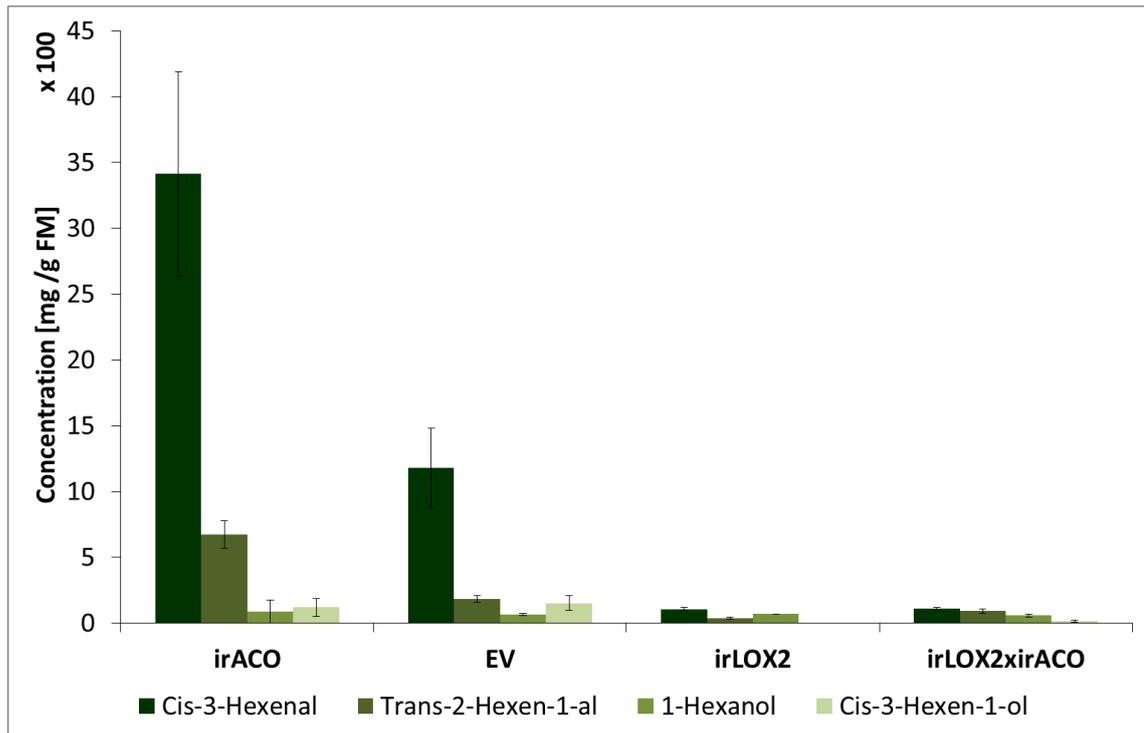


Figure 7: Concentration per biomass of internal *cis*-3-hexenal, *trans*-2-hexen-1-al, 1-hexanol and *cis*-3-hexen-1-ol in EV (A), irACO (B), irLOX2 (C) and irLOX2xirACO *N. attenuata* seedlings (D) in monoculture. Seedlings were harvested from boxes after 13 days. After special treatment internal GLVs were released from tissue and dissolved in CaCl₂ solution, equipped with 4-Hexanol internal standard and a 25 mm piece of PDMS. By PDMS absorbed internal GLVs were finally quantified by TD-GS-MS-analysis (n = 2 seedlings/box, 5 boxes per population*).

* only 1 population for all treatments with irACO

The internal GLV measurement underpinned the results from our silencing efficiency test (Figure 5). All genotypes which were not silenced in GLV synthesis (EV, irACO) had much higher GLV concentrations per biomass than the GLV-deficient genotypes (irLOX2, irLOX2xirACO). Especially *cis*-3-hexenol was elevated in EV and irACO, compared to irLOX2 and irLOX2xirACO. On the contrary the concentrations of 1-hexenol did not change very much.

5.2 Preliminary experiment

To check our designed experimental set-up and methods, an unreplicated test experiment was conducted at first. Leaf area expansion (LAE), photosynthetic rate, ethylene emission and finally biomass of seedlings were determined.

5.2.1 Cumulative leaf area expansion

We observed interesting differences in cumulative LAE in monocultures, expressed as daily leaf area relative to day one. Measurement started at day one, where seedlings were transferred to experimental set-up. Large differences were visible among the different seedling genotypes (Figure 8).

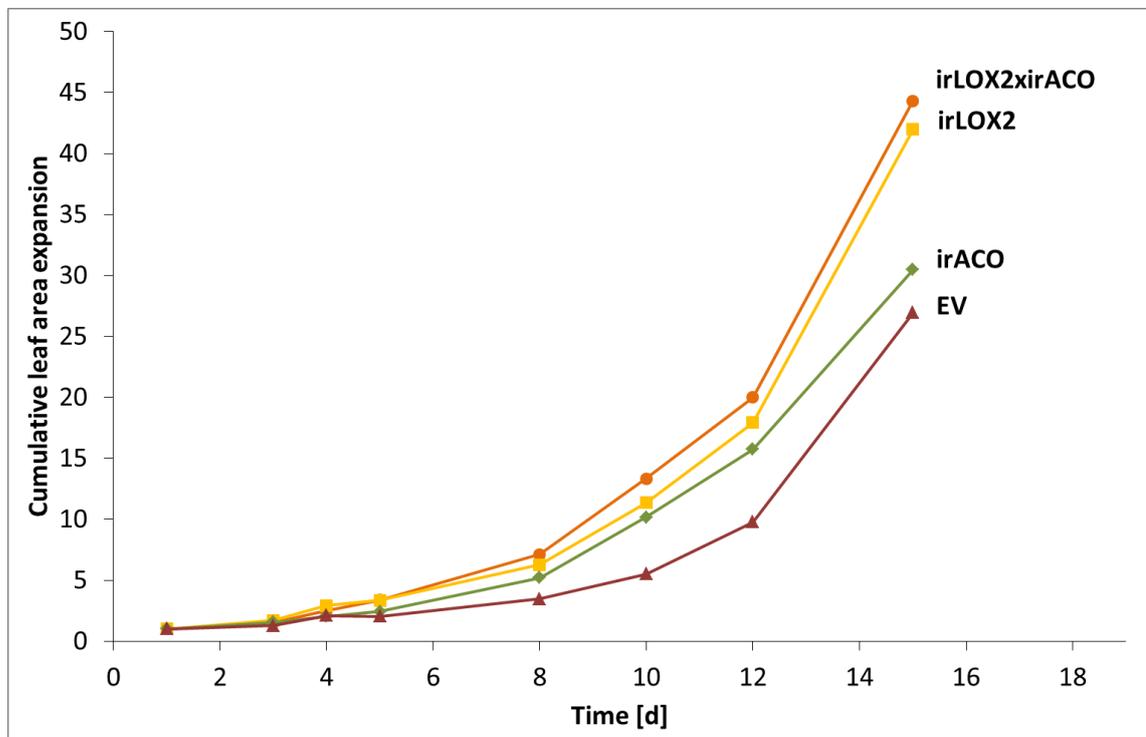


Figure 8: Cumulative LAE of different seedling genotypes in monoculture relative to day 1. Measurement started at day one, when seedlings were transferred to experimental set-up (n = 16 seedlings/box, 1 box per population type).

While leaf area of irLOX2xirACO seedlings increased by 45 times within 16 days, EV leaf area expanded only by 27 times, relative to day one. Expansion rates of the irACO and irLOX2 monocultures were intermediated between EV and irLOX2xirACO, but also differed greatly from each other. Leaf area of irLOX2 seedlings increased by about 42 times similar to irLOX2xirACO, while the irACO expansion rate was more similar to the EV.

Additionally, cumulative LAEs of individual seedling genotypes in combination with themselves or other genotypes were compared separately (Figure 9). Measurement started at day one, where seedlings were transferred to the experimental set-up.

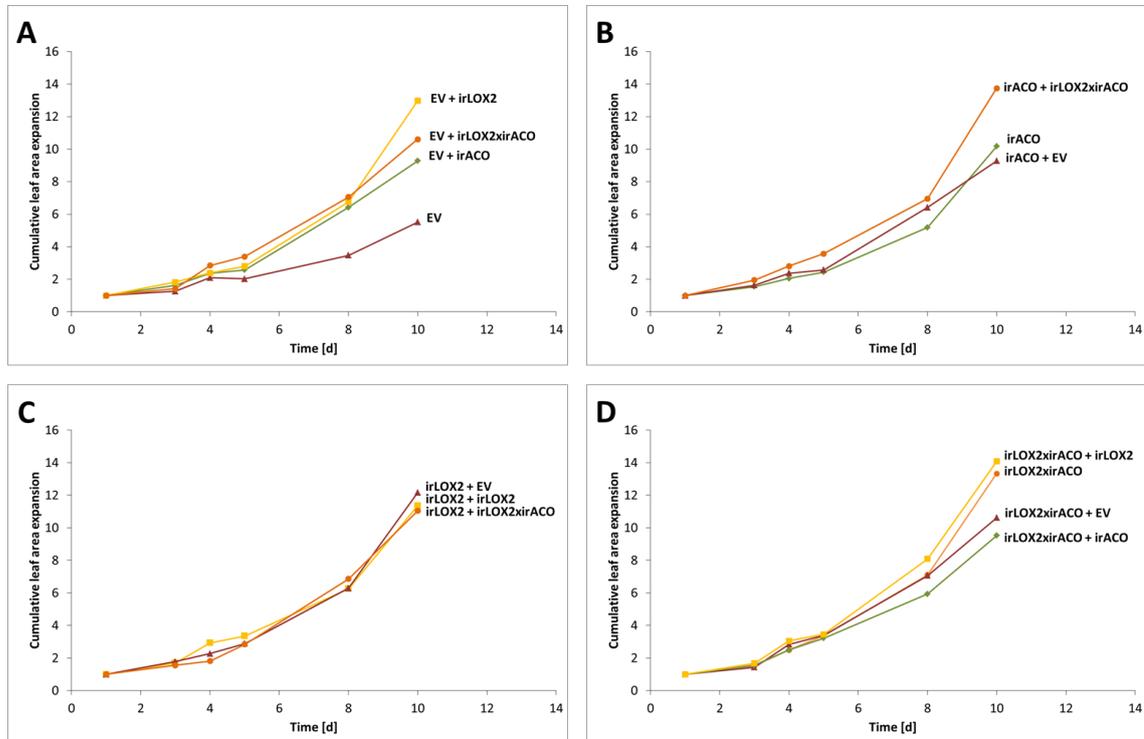


Figure 9: Cumulative leaf area expansion relative to day 1 of EV (A), irACO (B), irLOX2 (C) and irLOX2xirACO *N. attenuata* seedlings (D) in monoculture or 1:1 mixed cultures. Measurement started at day one, when seedlings were transferred to the experimental set-up (n = 16 seedlings/box, 1 box per population type).

We observed variation in LAE dependent on the competitor and its ability to produce ET and internal GLVs, as well as the ET and GLV production of the focal seedling. EV seedlings varied most within the different treatments. For example if EV seedlings were reared in populations with other genotypes having reduced ET or internal GLVs, seedling LAE was much greater than in monoculture (Figure 9A). All in all, the highest leaf area expansion rates could be seen in combination with all GLV-lacking genotypes. This effect was also clearly shown for the ET-deficient irACO seedlings (Figure 9B). These also had increased LAE when surrounded by seedlings without internal GLVs. LAE of irLOX2xirACO seedlings, deficient in ET and internal GLVs, showed the same pattern (Figure 9D). Only internal GLV-deficient irLOX2 seedlings did not display altered LAE in combination with different genotypes

5.2.2 Ethylene emission rate

The ET emission rates of whole seedling populations per shoot biomass were measured 18 days after transfer to experimental set-up, when seedlings were at their maximum size at the end of the experiment (Figure 10).

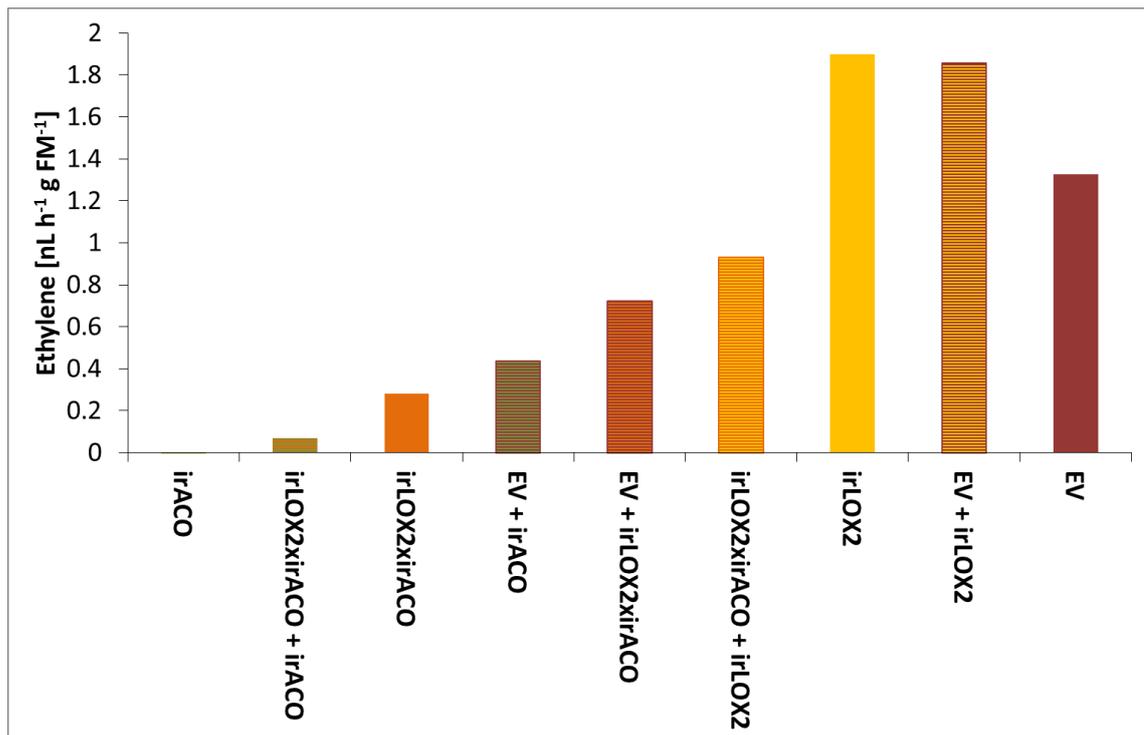


Figure 10: ET emission per shoot biomass of different *N. attenuata* seedling populations. Measurement was taken from closed boxes with controlled airflow 18 days after transfer of seedlings to the experimental set-up with a photo-acoustic trace gas sensor in continuous flow mode (n = 16 seedlings/box, 1 box per population type).

Overall, the measurement results from different populations reflected the emissions obtained from individual seedlings (Figure 6). The emission rates of both measurements had nearly the same amounts and ratio, dependent on the seedling genotypes. Consequently all seedling combinations with ET-deficient irACO or irLOX2xirACO genotypes had the lowest ET emission rates, whereas populations with EV or irLOX2 produced higher amounts. A clear correlation between population composition and measured ET emission rates was visible for all populations. For example the ET emission of the EV + irLOX2xirACO population was intermediate between the EV and the irLOX2xirACO monocultures. This was also shown for the EV versus irLOX2 or irACO populations, as well as for the irLOX2xirACO versus irLOX2 or irACO populations, which were intermediate between the monoculture emissions of the respective competitors.

5.2.3 Photosynthetic rate

The photosynthetic rates per leaf area were measured from whole populations directly from boxes at four different time points: 8, 10, 12 and 15 days after seedlings were transferred to the experimental set-up (Figure 11).

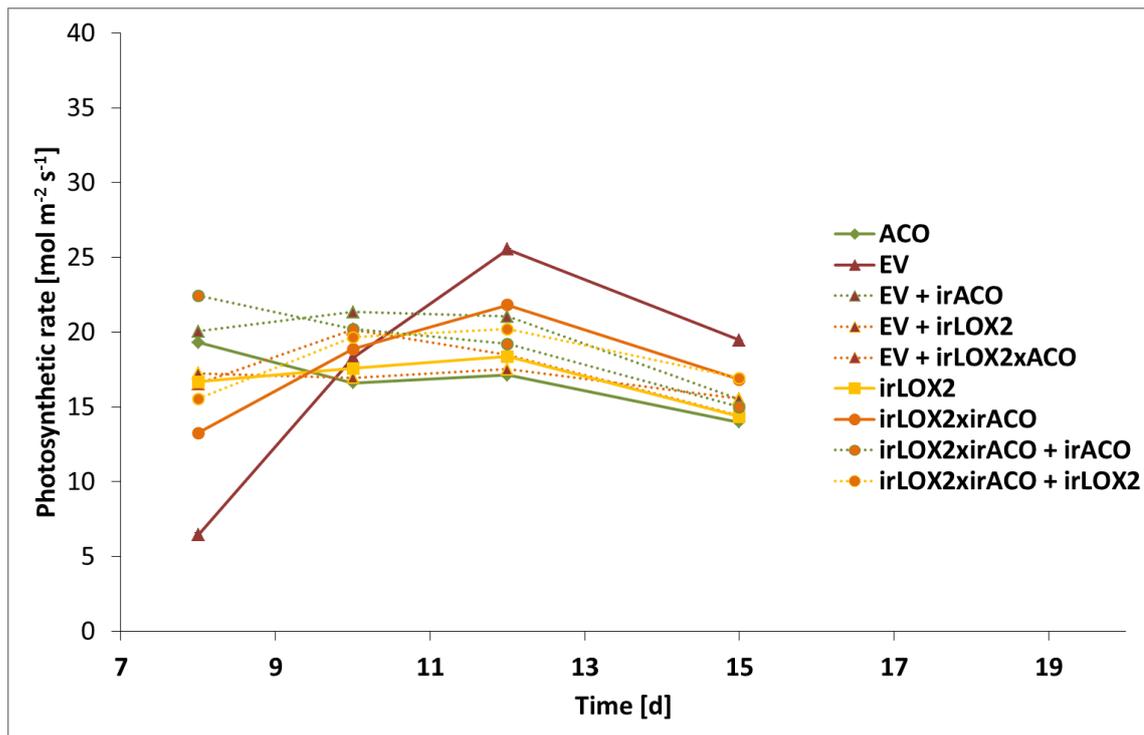


Figure 11: Photosynthetic rate per leaf area of different *N. attenuata* seedling populations. Measurements were conducted with an open-system portable infrared gas analyzer using a modified measurement chamber affixed directly to the top of boxes 8, 10, 12 and 15 days after seedlings were transferred to the experimental set-up (n = 16 seedlings/box, 1 box per population type).

For the majority of population types there was no considerable difference in photosynthetic rates. They had a near-linear trend over all time points with photosynthetic rates ranging between about 15 to 23 mol m⁻² s⁻¹. Only the EV monoculture had low rates at the first measurement. Five days after seedlings were transferred to boxes, EV had a photosynthetic rate of 6 mol m⁻² s⁻¹, one third that of the average of the other cultures. By day 12, the EV monoculture increased their photosynthetic rates to 25 mol m⁻² s⁻¹.

5.3 Main experiment with 5 replicates

Based on the primarily test experiment a bigger experiment with five replicates per population type was conducted three months later. In this experiment seedlings grew much faster. Consequently the individual seedlings could be distinguished only for six days after transfer, versus for ten days in the test experiment. While in the main experiment leaf area was up to 20 times bigger after six days, in the test experiment leaf area had amplified only 4-fold at that time point. Furthermore plants reached their maximum size three days earlier, why they were harvested sooner. Thus, ET emissions and shoot biomass were determined five days earlier. There were also germination problems with the irACO seedlings, why we had only one replicate for each irACO population type.

5.3.1 Cumulative leaf area expansion

Cumulative LAE of the different monocultures showed nearly the same pattern like in the first test experiment (Figure 12). Measurement started at day one, where seedlings were transferred to the experimental set-up.

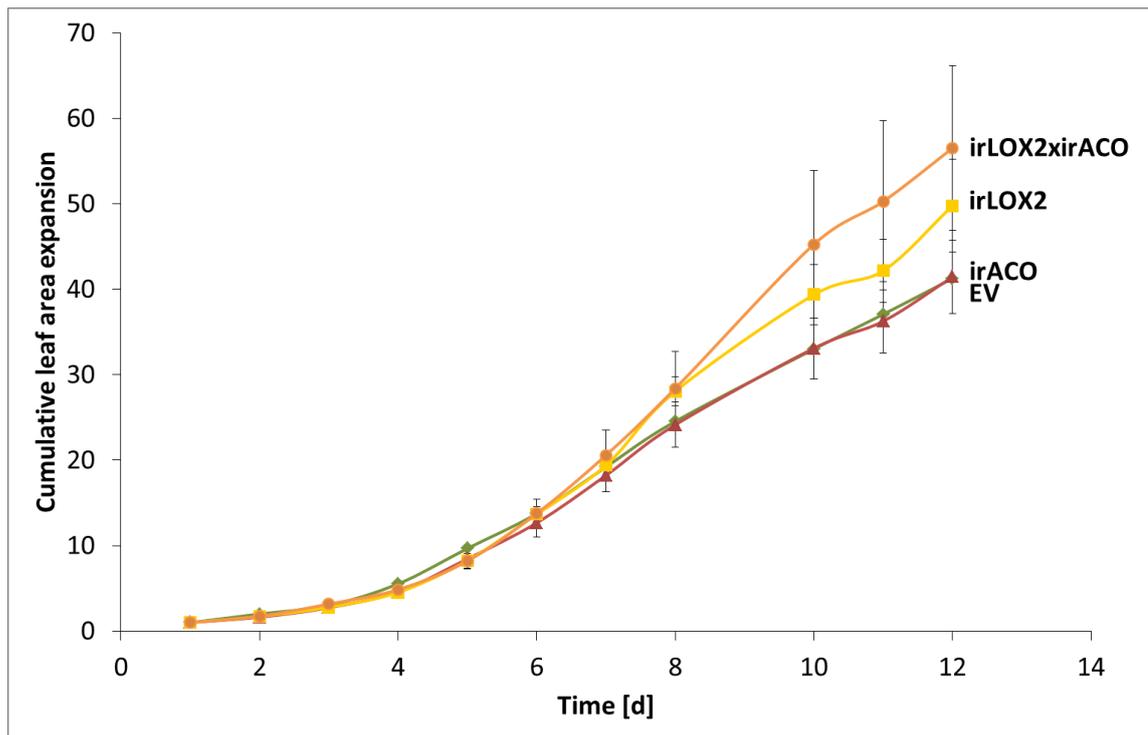


Figure 12: Cumulative LAE of different seedling genotypes in monoculture relative to day 1. Measurement started at day one, when seedlings were transferred to the experimental set-up (n = 16 seedlings/box, 5 boxes per population type*).

* only 1 population for all treatments with irACO

Again, the irLOX2xirACO monocultures had the highest LAE, whereas the EV monocultures had the lowest rates. Twelve days after transfer to the experimental set-up, leaf area per seedling of irLOX2xirACO was increased by 60 times, whereas EV seedlings were expanded only by 40

times relative to day one. LAE of the irLOX2 monocultures were intermediated between EV and irLOX2xirACO. The irACO seedlings had only slightly higher leaf area expansion rates than the EV seedlings. But the irACO data must be interpreted with caution as there was only one irACO monoculture.

In the replicated experiment, we did not observe large variation in cumulative leaf area expansion of individual seedling genotypes depending on the genotype of their neighbors (Figure 13). Measurement started at day one, when seedlings were transferred to the experimental set-up.

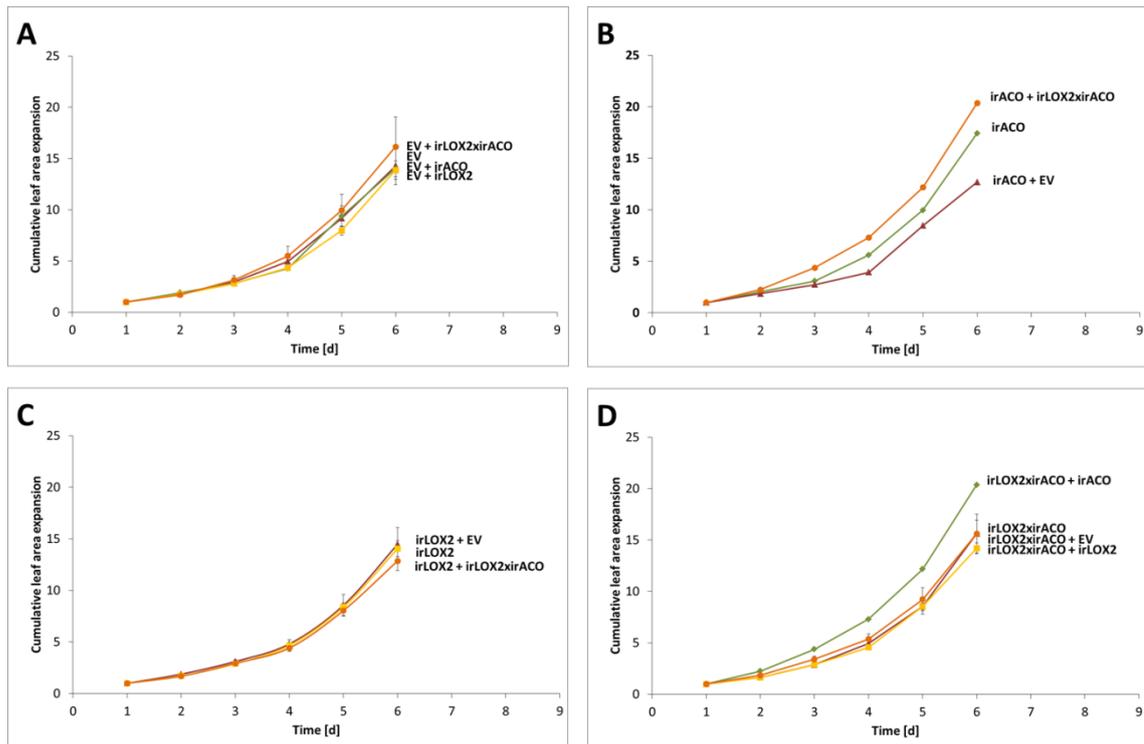


Figure 13: Cumulative leaf area expansion relative to day 1 of EV (A), irACO (B), irLOX2 (C) and irLOX2xirACO *N. attenuata* seedlings (D) in monoculture or 1:1 mixed cultures. Measurement started at day one, when seedlings were transferred to the experimental set-up (n = 16 seedlings/box, 5 boxes per population type*).

* only 1 population for all treatments with irACO

The inhibiting effect of the neighbor internal GLVs, shown in the test experiment, could not be repeated with more replicates. For EV (Figure 13A), irLOX2xirACO (Figure 13D) or irLOX2 (Figure 13C), where we had five replicates per population type, we did not observe variations in cumulative LAE dependent on the genotype of neighboring seedlings. But we found large variations in cumulative LAE among the respective replicates, e.g. for the EV monocultures (Figure 23). Only the irACO seedlings showed visible expansion differences, dependent on the neighboring plants (Figure 13B), which were comparable with the results from the test experiment (Figure 6B). The irACO seedlings seemed to be suppressed by neighbors that were able to produce GLVs (EV,

irACO), whereas cumulative LAE was elevated in combination with GLV-deficient genotypes (irLOX2, irLOX2xirACO). But due to large variations in cumulative LAE among the replicates, these results must be interpreted cautiously since the irACO populations were unreplicated.

5.3.2 Ethylene emission rate

In this experiment, ET emissions per shoot biomass of whole seedling populations (Figure 14) reflected mainly the emissions obtained from individual seedlings (Figure 6), as previously viewed in the test experiment (Figure 10). But different from previous experiments, ET emission rates were determined 12 days after transfer, when seedlings had their maximum size at the end of the main experiment.

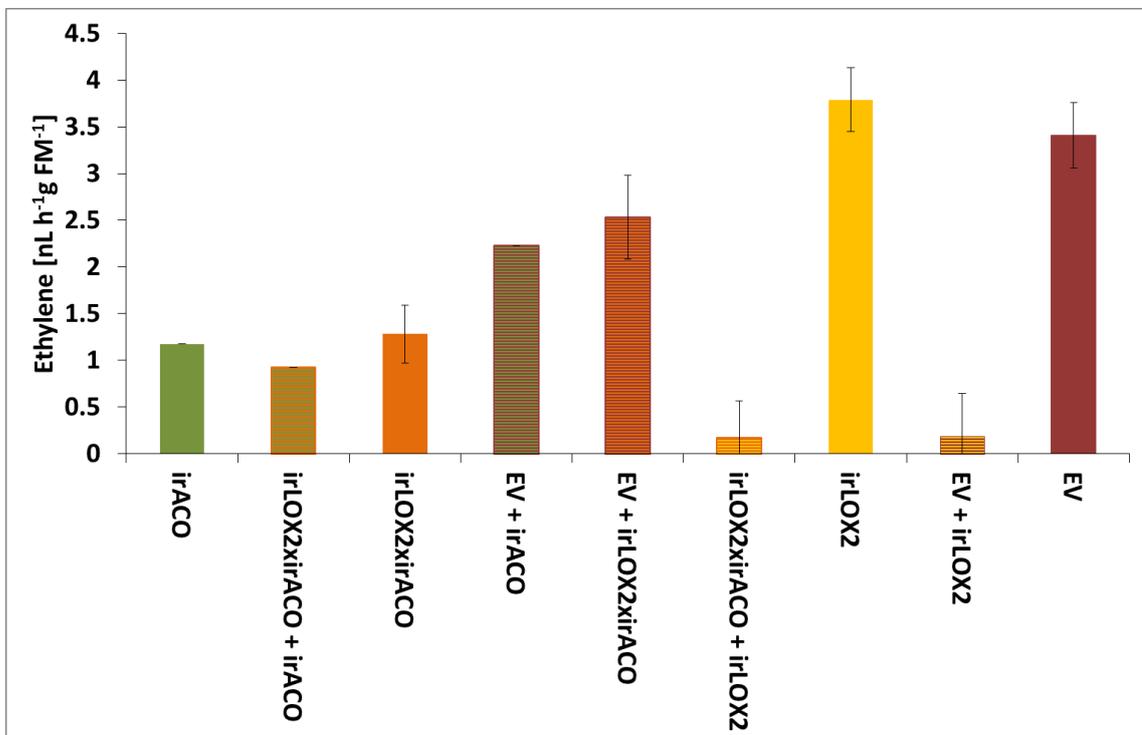


Figure 14: ET emission per shoot biomass of different *N. attenuata* seedling populations. Measurement was taken from closed boxes with controlled airflow 12 days after seedlings were transferred to the experimental set-up with a photo-acoustic trace gas sensor in continuous flow mode (n = 16 seedlings/box, 5 boxes per population type).

* only 1 population for all treatments with irACO

For two populations, the EV versus irLOX2 and the irLOX2xirACO versus irLOX2 population, which had wide variations and thus high standard errors, the measurements were not successful. Hence, they were neglected in further considerations. Apart from this all combinations with ET-deficient irACO or irLOX2xirACO seedlings had the lowest ET emission rates, whereas populations with EV or irLOX2 produced higher amounts. The correlation between population compositions and measured ET emission

rates, shown in the first test experiment, could be repeated. Again ET emissions of the EV + irLOX2xirACO populations were intermediated between the EV and the irLOX2xirACO monocultures. This could also be shown for the EV versus irACO populations, as well as for the irLOX2xirACO versus irACO populations. In this experiment we determined much higher ET concentrations than in previous measurements. For example the individual EV seedlings in the genotype screening emitted ET of about $1.6 \text{ nL h}^{-1} \text{ g FM}^{-1}$ (Figure 1), what was similar to the emissions rates of $1.4 \text{ nL h}^{-1} \text{ g FM}^{-1}$ per seedling of the EV monoculture in our preliminary experiment. However, in this experiment we had emissions of $3.5 \text{ nL h}^{-1} \text{ g FM}^{-1}$ per seedling in the EV monocultures.

5.3.3 Photosynthetic rate

Gas exchange measurements of whole populations were taken to see whether the composition of populations influences their energy metabolism and thus photosynthetic rates per leaf area. Measurements were taken from whole populations directly from boxes at three different time points: 5, 8, and 11 days after seedlings were transferred to the experimental set-up (Figure 15).

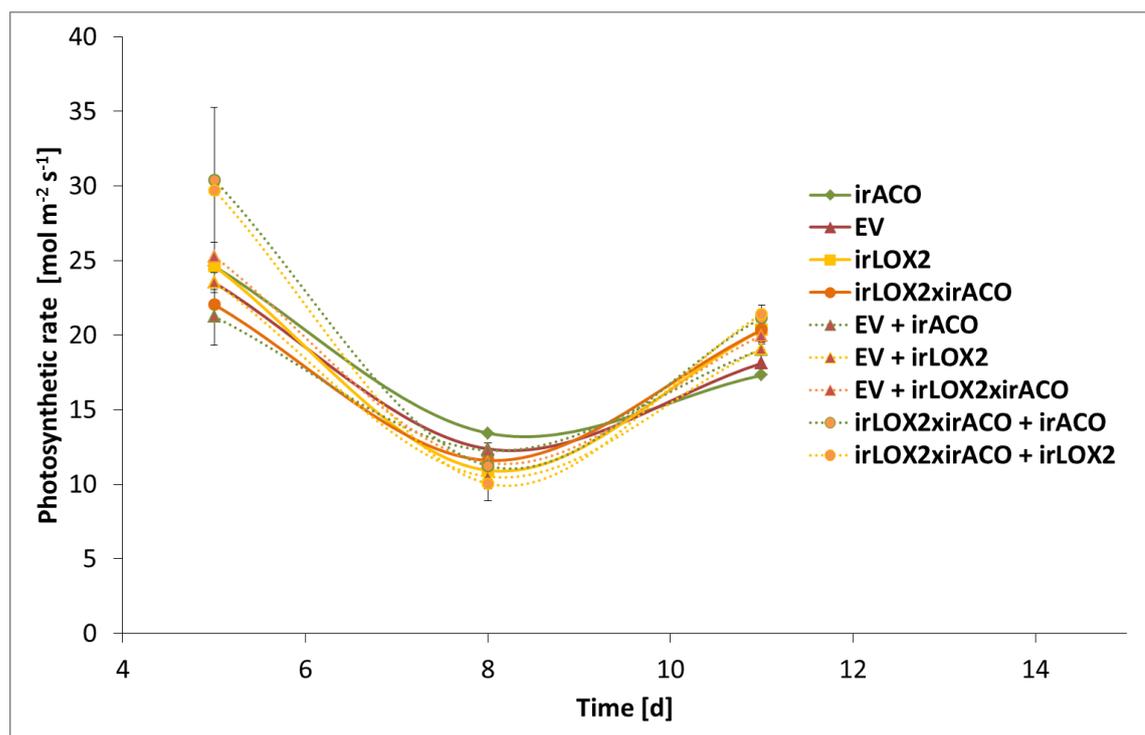


Figure 15: Photosynthetic rate per leaf area of different *N. attenuata* seedling populations. Measurements were conducted with an open-system portable infrared gas analyzer 5, 8 and 11 days after seedlings were transferred to boxes (n = 16 seedlings/box, 5 boxes per population type*).

* only 1 population for all treatments with irACO

All population types had nearly identical photosynthetic rates. They all showed the same dynamic over all time points with photosynthetic rates ranging between about 10

to $30 \text{ mol m}^{-2} \text{ s}^{-1}$. The highest photosynthetic rates with about 20 to $30 \text{ mol m}^{-2} \text{ s}^{-1}$ was measured five days after seedlings were transferred to boxes. At day eight all photosynthetic rates were slightly dropped to an average of $13 \text{ mol m}^{-2} \text{ s}^{-1}$. Three days later photosynthetic rate was elevated again, to circa $20 \text{ mol m}^{-2} \text{ s}^{-1}$.

5.3.4 Internal GLVs

Internal GLV concentrations per shoot biomass of the genotypes in monoculture differed greatly from each other (Figure 7). All monocultures that were not silenced in GLV synthesis (EV, irACO) had much higher GLV concentrations per biomass than the GLV-deficient genotypes (irLOX2, irLOX2xirACO). Especially *cis*-3-hexenol was elevated in EV and irACO, compared to irLOX2 and irLOX2xirACO, whereas the concentrations of 1-hexenol didn't alter very much. It was odd that the irACO seedlings showed much higher *cis*-3-hexenol and *trans*-2-hexen-1-al concentrations than the EV seedlings.

In addition internal GLV concentrations per shoot biomass of individual seedling genotypes in combination with themselves or other genotypes were compared separately (Figure 16). For the measurement seedlings were harvested 13 days after they were transferred to the experimental set-up.

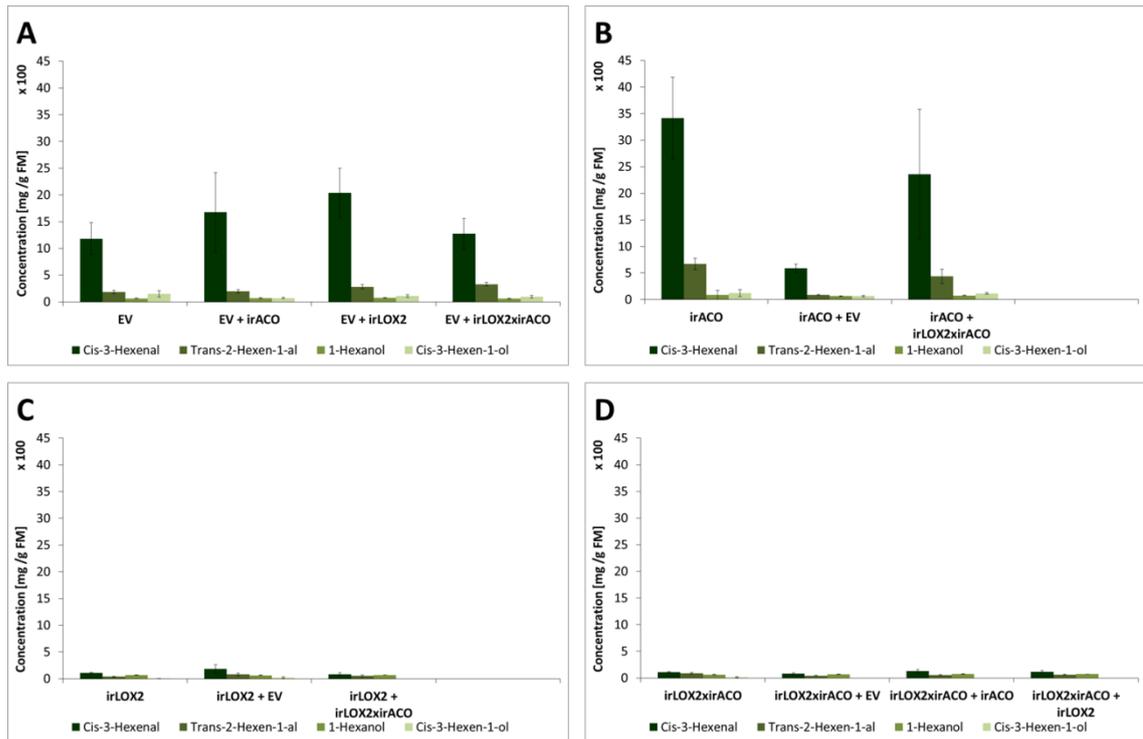


Figure 16: Concentration per biomass of internal *cis*-3-hexenal, *trans*-2-hexen-1-al, 1-hexanol and *cis*-3-hexen-1-ol in EV (A), irACO (B), irLOX2 (C) and irLOX2xirACO *N. attenuata* seedlings (D) in monoculture or 1:1 mixed cultures. Seedlings were harvested 13 days after transfer to the experimental set-up. After special treatment internal GLVs were released from tissue and dissolved in CaCl_2 solution, equipped with 4-Hexanol internal standard and a 25 mm piece of PDMS. By PDMS absorbed internal GLVs were finally quantified by TD-GS-MS-analysis (n = 10 seedlings per population).

As previous shown for the monocultures, seedlings that weren't silenced in GLV biosynthesis had overall much lower internal GLV concentrations, than GLV-deficient seedlings. For the irLOX2, the irLOX2xirACO and the EV seedlings little variance among the different populations was found. The irACO seedlings had increased concentrations of *cis*-3-Hexenal, *trans*-2-Hexen-1-al and *cis*-3-Hexen-1-ol in monoculture and in combination with irLOX2xirACO. On the contrary in combination with EV, concentrations were relative low. Production of internal GLVs in irACO seedlings seemed to be suppressed by ET producing neighbor plants. But since there was only one replicate for all irACO treatments, these correlations were not reliable. Nevertheless by comparing the internal GLV concentrations of the irACO seedlings with their cumulative LAE, there were apparent similarities. While both cumulative LAE and internal GLV concentrations were low for irACO in population with EV, both parameters were elevated in population with irLOX2xirACO and irLOX2.

5.4 External GLV supplementation

Parallel to our main experiment with five replicates, GLVs were added to some extra EV and LOX2 monocultures (n = 5). GLV supplementation started 24 h after transfer to the experimental set-up on the second day of the main experiment.

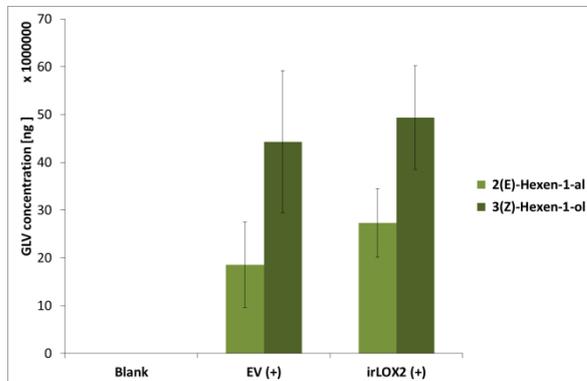


Figure 17: Amounts of *trans*-2-hexen-1-al (2-(E)-Hexen-1-al) and *cis*-3-hexen-1-ol (3-(Z)-Hexen-1-ol) trapped over 24 h after GLV supplementation from EV and irLOX2 *N. attenuata* monocultures, as well as from blank boxes without GLV supplementation. Nightly 0.1 mL of with GLVs concentrated (+) or pure lanoline paste (-) was added (n = 16

Supplementation was conducted nightly at 9 p.m. Over 24 h amounts of about 50 mg of *cis*-3-Hexen-1-ol and about 25 mg of *trans*-2-hexen-1-al were trapped from the GLV-supplemented box headspaces (Figure 17). From blank boxes with pure lanolin paste no GLVs were detected. Furthermore leaf area expansion, photosynthesis, ET emission and biomass were determined and checked against EV and irLOX2 monocultures without external GLVs.

5.4.1 Daily leaf area expansion

We observed interesting differences in daily LAE among the respective population types of the EV and irLOX2 monocultures (Figure 18). Daily LAE was expressed as daily leaf area increase relative to day one. Leaf area measurements started at day one, where seedlings were transferred to the experimental set-up, whereas GLV supplementation begun at day two.

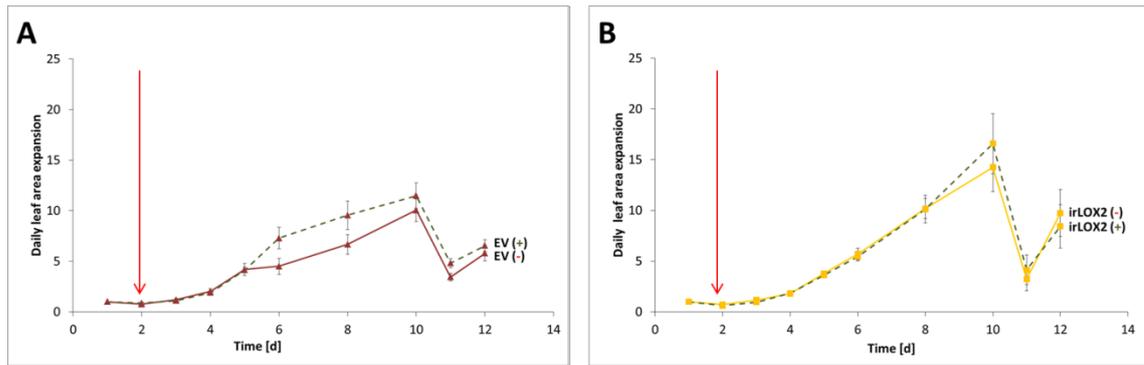


Figure 18: Daily leaf area expansion of EV (A) and irLOX2 *N. attenuata* seedlings (B) in monoculture with (+) and without GLV supplementation (-). Nightly 0.1 mL of with GLVs concentrated or pure lanoline paste was added to all populations starting two days after transfer to boxes. Measurement started at day one, when seedlings were transferred to experimental set-up (n = 16 seedlings/box, 5 boxes per population).

We observed considerable variations in daily LAE among the EV monocultures with and without GLV supplementation (Figure 18A). Six days after transfer to boxes, plants developed different daily LAE. While GLV-supplemented EV seedlings, still elevated their daily LAE from day 5 to day 6, plants without external GLV addition stagnated. Prior LAE of all EV monocultures was similar. In contrast there was no variation between GLV-supplemented and not-supplemented irLOX2 monocultures (Figure 18B). All irLOX2 populations elevated their daily LAE until the tenth day. Altogether irLOX2 monocultures reached higher daily LAE as well as higher cumulative LAE than the EV monocultures (Figure 12). Eleven days after start of the experiment, daily LAE of all population types had a sharp drop, but started to elevate again one day later.

5.4.2 ET emission rate

The ET emission rates per shoot biomass of whole seedling populations were measured 12 days after transfer to the experimental set-up, when seedlings were at their maximum size (Figure 19).

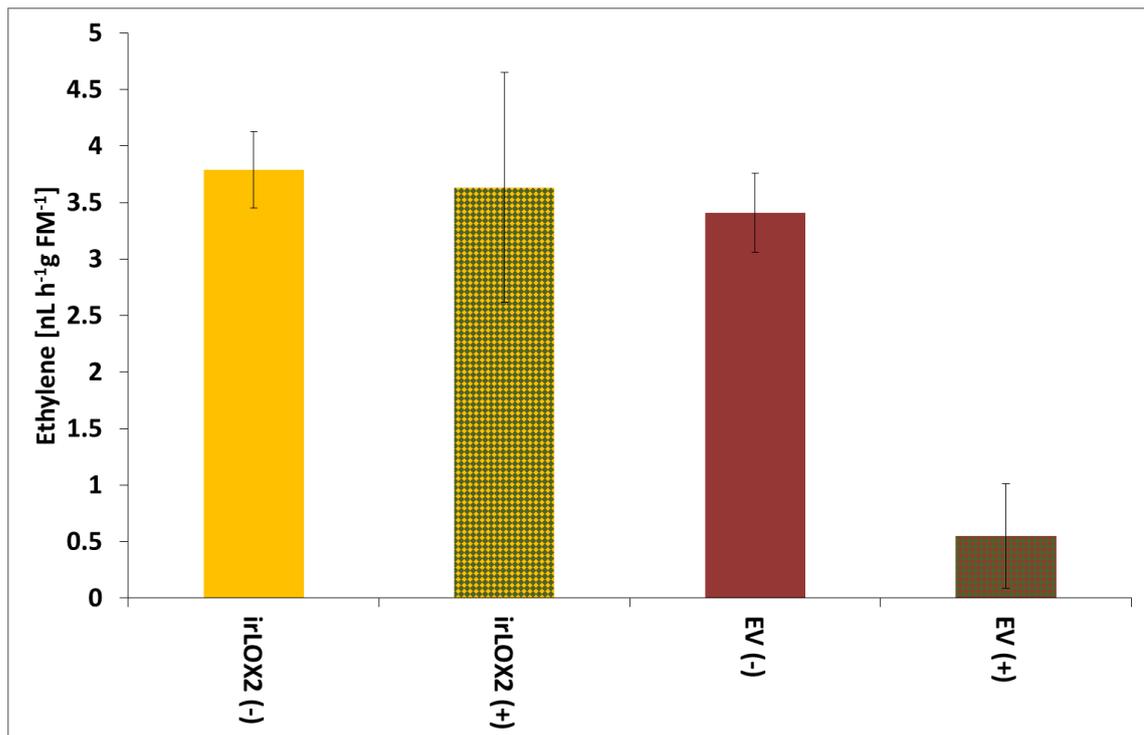


Figure 19: ET emission rates per shoot biomass of irLOX2 and EV seedlings in monoculture with (+) and without GLV supplementation (-). Nightly 0.1 mL of with GLVs concentrated or pure lanoline paste was added. Measurements were taken from closed boxes with controlled airflow 12 days after transfer of seedlings to the experimental set-up with a photo-acoustic trace gas sensor in continuous flow mode (n = 16 seedlings/box, 5 box per population).

For both irLOX2 monoculture population types similar ET emissions were observed, whereas the ET emissions of the EV monocultures varied deepened on external GLV amounts. Whereas EV seedlings without external GLVs had ET emission rates of about $3.5 \text{ nL h}^{-1} \text{ g FM}^{-1}$, emission rates of cultures with GLV supplementation were seven-fold lower. Apart from this ET productions of normal EV and irLOX2 monocultures were more or less the same.

5.4.3 Internal GLV concentrations

Internal GLV concentrations per shoot biomass of the respective population types of EV and irLOX2 monocultures were observed after seedlings were reared for 13 days in the experimental set-up (Figure 20).

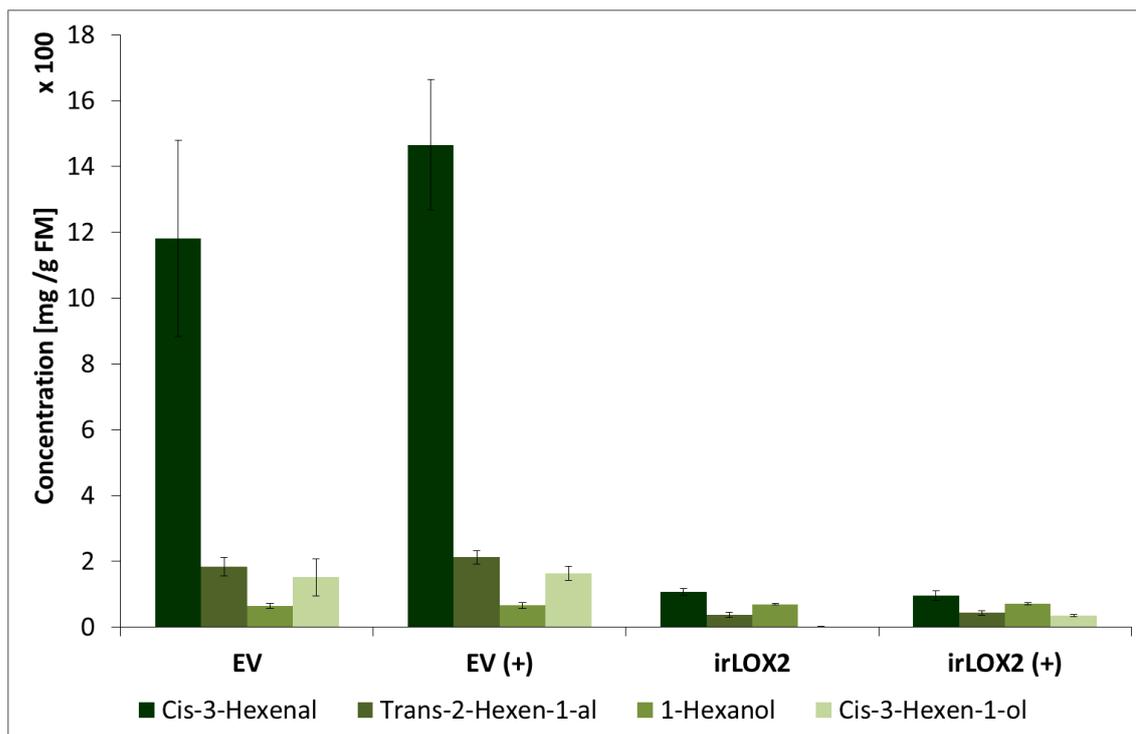


Figure 20: Concentration per biomass of internal *cis*-3-hexenal, *trans*-2-hexen-1-al, 1-hexanol and *cis*-3-hexen-1-ol in EV and irLOX2 *N. attenuata* seedlings from monocultures with (+) and without GLV supplementation (-). Nightly 0.1 mL of with GLVs concentrated or pure lanoline paste was added. Seedlings were harvested 13 days after transfer to boxes. After special treatment internal GLVs were released from tissue and dissolved in CaCl₂ solution, equipped with 4-Hexanol internal standard and a 25 mm piece of PDMS. By PDMS absorbed internal GLVs were finally quantified by TD-GS-MS-analysis (n = 10 seedlings per population).

For both the irLOX2 and the EV monocultures seedlings internal GLV concentrations differed little among the populations with and without GLV supplementation. As previous shown EV seedlings had much higher internal GLV concentrations than tirLOX2 seedlings.

6. Discussion

6.1 Population level effects of GLV and ET production in seedlings

Our fundamental question was whether GLVs and ET synergistically function in seedling competition. They are supposedly correlated over reactive oxygen species (ROS) that result from photosynthetic electron transport and metabolism. ROS are toxic in high amounts, which is why their production is regulated over specific enzymes like NADPH oxidases and respiratory burst oxidase homologues (RBOHs). Apart from this, in combination with other signals, they regulate many biological processes, including growth, development or response to abiotic and biotic stresses (Baxter, Mittler, & Suzuki, 2014). ROS have an important role in the compartment-specific-redox-regulated signaling system (Foyer & Noctor, 2013), which transports information from compartments to the nucleus in order to regulate gene expression in cells, but also conducts signal propagation between cells over ROS waves (Dubiella et al., 2013). Previous studies by Steinhorst & Kudla (2013) found a direct connection between the ROS and Ca^{2+} signaling pathways in cell-to-cell communication. Ca^{2+} is an important second messenger that is released in consequence of biotic and abiotic stresses. Ca^{2+} fluxes over the membrane into the cell and elevates cytosolic Ca^{2+} concentrations. Specific cytosolic Ca^{2+} sensor proteins perceive defined Ca^{2+} signals and transmit them into cytosolic signals, which lead to activation of RBOHs and generation of ROS. As a consequence, Ca^{2+} channels of the neighboring cell are activated whereby the signal is propagated with a high velocity. In plants there is a “complex interface between the redox and hormone-signaling pathways that allows precise control of plant growth and defense in response to metabolic triggers and environmental cues” (Foyer & Noctor, 2013). For instance, high light and thus alteration of redox conditions induces expression of ethylene-regulated genes, as well as production of ROS and the ET precursor ACC (Baxter, Mittler, and Suzuki 2014). In addition, ET biosynthesis is modulated through positive regulation by ROS producing RBOH proteins and negative regulation by constitutive triple response proteins. Besides ET, also the oxylipin pathway, which *inter alia* results in GLVs, is partly regulated through ROS. Abiotic stresses, like high light treatments, as well as wounding, result in stronger HPL expression (Xiao et al., 2012). It is assumed that HPL is regulated through the ROS signaling system, dependent on the redox state of the plastids. HPL is a stress-inducible enzyme, which converts linoleic acid 13-hydroperoxide (13HPOT), provided by LOX2 in *N. attenuata*, to *cis*-3-hexenal and its derivatives. 13HPOT can't accumulate to high amounts in cells, because it strongly interacts with the cell membrane phospholipids and components. That increases cell membrane fluidity, which leads to cell death (Tran Thanh et al., 2007). Elevated 13HPOT amounts and thus increased membrane fluidity could supposedly result in cytosolic Ca^{2+} influx, which leads to ROS and HPL synthesis.

In consequence 13HPOT conversion is controlled through dynamic composition and decomposition of HPL, controlled through ROS. Hence, GLV production by HPL is induced through ROS, whereas ET correlates with ROS, to direct plant growth and defense. Thus, there could be a link between ET and GLV biosynthesis, which could synergistically influence seedlings behavior in competition situations.

In the experiments we used four transgenic genotypes of *N. attenuata* which were deficient in GLVs (irLOX2), ET (irACO), both (irLOX2xirACO) or without silencing (EV). The silencing was demonstrated in the silencing efficiency test, where we showed reduced expression of *NaACO* or *NaLOX2* in the respective silenced genotypes (Figure 6). That was finally confirmed by determination of the respective internal GLV concentrations and ET emissions of these genotypes (Figure 7; 8).

The experiment was conducted twice. In the preliminary experiment we had only one replicate for all populations to test the experimental set-up and determine whether we might observe interesting differences in a replicated experiment. Finally in the main experiment the observations from the preliminary test should have been reproduced with five replicates. Unfortunately we had germination problems for the irACO plants, which is why we had only one replicate for all populations with irACO. The germination problems in the main experiment were caused by seasonal germination dependency of *N. attenuata*. During September and February germination capability of seeds is reduced due to dormancy. The main experiment was conducted in October, whereas the preliminary experiment in August. For all genotypes we saw reduced germination rates starting from September. Furthermore seedlings grew faster in the main experiment in October than in the earlier investigations. The reason for different growth velocities in both experiments is unclear, since seedlings always grew under similar conditions.

In both, the preliminary test (n = 1) and the main experiment (n = 5) the genotypes were reared in monocultures or 1:1 mixed cultures. Independent of the genotypes, all populations had similar photosynthetic rates, which were approximately constant (Figure 11, 15). Thus, different ET emissions or internal GLV concentrations in seedling populations did not influence plants' photosynthetic rate. Photosynthesis is a process to convert light energy into chemical energy, which can be further released for metabolism, growth, defense, competition and reproduction of plants. Thus, all genotypes gathered the same energy amounts, since all plants had similar light conditions in our experimental set-up. However, how plants invest their available energy is dependent on their genotype, age, conditions and environmental factors.

For ET emissions of the populations in both, the preliminary and the main experiment, similar observations were made, as for the individual seedlings in the genotype screening (Figure 10, 14). All monocultures of ET-deficient genotypes (irACO, irLOX2xirACO) had very low ET emission rates, compared to the monocultures with ET-synthesizing genotypes. The ET concentrations of the mixed cultures were always

intermediated between the respective monocultures. Hence, seedling ET emissions seemed to be independent of the genotype of neighboring seedlings. However, there was striking evidence that younger seedlings produce higher ET amounts than older ones. In our main experiment ET emissions were measured at 12 days, whereas in the preliminary experiment and the genotype screening (Figure 6), ET was measured 18 days after seedlings were transferred to boxes. At the earlier time point much higher concentrations were observed. This is interesting since the phytohormone ET is *inter alia* involved in growth and germination processes (Bleecker & Kende, 2000). With increasing age, plants shift their energy supply from germination and growth promoting processes to other important mechanisms, like reproduction or defense.

The observed internal GLV concentrations in the main experiment also reflected the genotype silencing (Figure 16). Overall the GLV-deficient genotypes (irLOX2 and irLOX2xirACO) had similar low internal GLV concentrations in combination with themselves or other genotypes. In contrast, genotypes which could express LOX2 had overall much higher internal GLV amounts. But whereas EV had always similar internal GLV concentration independent of the neighbor genotype, the internal GLV concentrations in the irACO seedlings varied in the populations. The irACO seedlings produced less GLVs in combination with EV plants and higher amounts in populations with ET-lacking genotypes. But since all populations with irACO were unreplicated in the main experiment, the variations of internal GLV amounts in irACO could also be due to normal deviations among the seedlings. However, if we compare the internal GLV concentrations of all EV monoculture replicates and the irACO monoculture, the EV seedlings had always lower amounts than irACO (Figure 22). This finding indicates that the observed effects are trustworthy. Thus, we conclude that ET suppresses GLV production in ET-deficient plants, whereas GLV biosynthesis is elevated in populations without external ET.

In cumulative LAE great variances were detected for monocultures in both, the main experiment (n = 5) and the preliminary test experiment (n = 1; Figure 8, 12). In monoculture populations, plants have no disadvantage from missing opportunities to synthesize or sense ET and GLVs. Thus, seedlings in monocultures can grow without risk of being suppressed by a stronger competitor. In combination with superior competitors, genotypes that cannot produce GLVs or ET might suffer a disadvantage. Differences in LAE among the genotypes occur due to dissimilar carbon and energy usage. Whereas the irLOX2xirACO seedlings have no energy investment in ET or GLV production, EV seedlings release much energy in these pathways. Thus, EV seedlings had less cumulative LAE than the other genotypes, while irLOX2xirACO had the highest cumulative LAE. Cumulative LAE of the irACO and irLOX2 monocultures, silenced in only one pathway each, was intermediate between the EV and irLOX2xirACO monoculture.

In contrast to monoculture populations, in mixed populations the inferior competitor is suppressed by the stronger partner. By comparing LAE of individual seedling genotypes in combination with themselves or 1:1 mixed with other genotypes, we had reverse results for both experiments (Figure 9, 13). Whereas the presence of GLV-producing genotypes seemed to suppress LAE of their neighbors in the preliminary experiment ($n = 1$), these observations were not reproduced in the main experiment. We did not find large variations in leaf area dependent on the neighbor plants' genotype. Only the irACO seedlings showed the same suppression by GLV-synthesizing competitors in both experiments. But we are skeptical towards these results, since the irACO populations were unreplicated in both investigations. Furthermore if we compare LAE of all EV monoculture replicates, we observe great variations among the different LAE rates (Figure 23). In addition, LAE of the irACO monoculture was intermediated among the LAEs of the EV monocultures and could not be distinguished. Accordingly, the replicated populations of the main experiment showed the most authentic results, since we found great variations among all replicates. Thus, internal GLVs and ET emissions of neighbor plants were not shown to affect seedlings' LAE in our investigations.

6.2 Impact of external GLV supplementation

In addition to our main question, whether GLVs and ET synergistically function in seedling competition, we asked if external GLV accumulation influences seedling growth. Previous studies on tomato plants demonstrated that supplementation with GLVs, like *cis*-3-hexenal, *trans*-2-hexenal and *cis*-3-hexenyl-acetate, leads to a strong depolarization of the plasma membrane and Ca^{2+} influx. This results in activation of NADPH oxidase and production of ROS (Zebelo, Matsui, Ozawa, & Maffei, 2012), which, as described earlier, is known to be involved in many biological processes in response to stress. In addition, 2006 Paschold, Halitsch and Baldwin discovered 11 genes that reacted significantly to exposure to a synthetic GLV mix, consisting of GLVs typically released by *N. attenuata* after wounding (hexanal, *trans*-2-hexenal, hexanol, *trans*-2-hexenol, *cis*-3-hexenol and *cis*-3-hexenyl acetate). The most of these volatiles were also included in our supplemented mix. Two genes that were induced are known to be involved in stress regulation and one in photosynthesis, the rest is to date unknown. That is consistent since Ca^{2+} influx and thus ROS synthesis also occur in response to biotic and abiotic stresses, like high light or wounding. Furthermore they observed genes that were only expressed without GLV supplementation, whereas plants exposed to GLVs did not induce these genes. That indicates that *N. attenuata* seedlings are able to sense external GLVs and react to them by altering their gene expression. The induction of stress-regulated genes in response to external GLVs indicates that GLV supplementation is a cue of biotic or abiotic stress, which mostly results from wounding. Abiotic stress factors are high light, frost, drought and wind, whereas insect

herbivory and pathogen infestation are biotic factors. In general GLVs are not released by plants under normal conditions. Nevertheless GLVs are permanently synthesized and degraded in a diurnal manner (Y. Joo and M. Schuman, unpublished). Thus, plants constantly contain internal GLVs in altering amounts dependent on their daily rhythm. However, wounding, induces the release of GLVs in high amounts as a product of enzymatic activity of HPL (Bate & Rothstein, 1998). HPL is supposedly activated through Ca^{2+} flux in cells and ROS in response to wounding. Thus, environmental GLV accumulation could be a sign of stress factors for eavesdropping plants. Previous observations showed that GLV perception induces several genes known to be involved in plant defense. For instance Engelberth (2004) demonstrated that GLV exposure of *Zea mays* plants induced JA production and VOC emission. Besides GLVs, jasmonates (JAs) are produced over the oxylipin pathway by 13-LOX enzymes, as described in the introduction. JAs are involved in the mediation of herbivore resistance through BVOC releases and induction of direct defense traits, like synthesis of nicotine and activation of trypsin proteinase inhibitors (Halitschke & Baldwin, 2003). In addition, *Arabidopsis* seedlings also showed expression of several defense-related genes of the LOX pathway in response to *trans*-2-hexenal treatment (Bate & Rothstein, 1998). In conclusion eavesdropping plants interpret external GLVs superficial as signal of lurking herbivory, since perceiver react with defense response. However, Paschold, Halitsch and Baldwin (2006) did not observe transcript accumulation of the herbivore-inducible *NaLOX3* gene, which channels the JA biosynthesis, in *N. attenuata*. In addition, no increase in JAs was detected in the GLV-receiving plants. In contrast when seedlings were exposed to blends emitted by *Manduca sexta* infested plants, they showed specific JA-correlated defense mechanism. Hence, *N. attenuata* plants distinguish between different perceived volatile blends and respond with specific reactions. The results of Paschold, Halitsch and Baldwin indicate that GLV exposure does not directly prime JA induced defense mechanisms in *N. attenuata* in contrast to observations on *Arabidopsis* and *Zea mays*. Nevertheless additional observations of *N. attenuata* exposed to BVOC blends of clipped *Artemisia tridentata*, followed by infestation with *Manduca sexta*, showed less damage due to herbivory and higher mortality of feeding insects, compared to plants without previous exposure (André Kessler, Halitschke, Diezel, & Baldwin, 2006). In addition, BVOC releases of GLV-supplemented corn plants were elevated during subsequent herbivory (Engelberth et al., 2004). Thus, exposure to damaged plants induces priming for later infestation and finally an elevated defense response. Besides defense, GLVs could also indicate potential competitors. In consequence, altered gene expression in reaction to external GLVs could additionally induce phenotypical modifications or other competition traits, in order to successfully compete with neighbors.

To test the effect of external GLVs on plants we additionally added a synthetic GLV mix to some monocultures of GLV-deficient *irLOX2* seedlings and also GLV-synthesizing EV seedlings (Figure 17). The GLV mix was similar to GLV blends emitted by *N. attenuata* after herbivory. In contrast to natural populations without any supplementation, divergent LAE were found for GLV-supplemented EV monocultures

versus control. External GLV addition to EV monocultures triggered daily LAE of EV plants, while *irLOX2* monocultures didn't react to GLV supplementation. The phenotypical modification of GLV-supplemented EV seedlings developed after an adaptation period of four days. In conclusion plants that express LOX2, respond to external GLVs by elevated LAE rates. Thus, the increase in LAE in response to GLV supplementation is dependent on the *NaLOX2* gene.

Since the *irLOX2* monoculture did not react to external GLVs with elevated LAE, we supposed that expression of LOX2 might be essential to develop a primed defense response. We thus hypothesized that responses in GLV-supplemented EV seedlings are accompanied by higher internal GLVs. However, all EV monocultures had similar internal GLV concentrations independent of the presence of external GLVs (Figure 20). This was also observed for the *irLOX2* monocultures, whereas their internal GLV amounts were much smaller. Since we did not evidence a higher LOX2 expression in the supplemented EV monocultures we assume that LOX2 silencing in *irLOX2* might be accompanied with silencing of LOX2 downstream metabolites, which are responsible for GLV perception in plants. Hence, *irLOX2* did not perceive external GLVs and thus did not react to them.

Consistent with their similar daily LAE, all *irLOX2* monocultures showed similar ET emission rates, whereas ET emissions of the EV populations varied (Figure 19). GLV-supplemented EV monocultures had seven times lower ET emissions than non-supplemented monocultures. There seems to be a trade-off between LAE and ET emission in EV plants. Perhaps the energy and carbon consumption for elevated LAE is taken from ET biosynthesis, by suppressing ET-related genes. Thus, a stronger LAE performance, in consequence to external GLVs, is correlated with less ET emissions due to gene regulation.

In a previous study GLV supplementation was found to influence a gene involved in photosynthesis (Paschold et al., 2006). Nevertheless we did not detect differences in photosynthetic rate among the monocultures with and without GLV supplementation. The photosynthetic rate was nearly constant over all measured time points and thus, was not influenced by external GLVs.

6.3 Conclusion

In the work reported here, we did not find evidences for our hypothesis that ET and GLVs synergistically influence seedlings' competition. The investigated genotypes, with varying internal GLV concentrations and ET emissions, did not show visible adjustments to different neighbor genotypes. Our indicator for response to different neighbor genotypes was seedlings' LAE. LAE was nearly stable within each genotype regardless of neighbor genotypes, except in the unreplicated populations. In the

preliminary test experiment (n = 1) we found striking differences. But that was due to normal variance among seedlings of one genotype, since we had only one replicate per population type.

In contrast to the natural populations, we observed an effect in LAE in populations with additional GLV supplementation. The EV genotype elevated its daily LAE in GLV-supplemented monocultures. Hence, we assume that GLV concentrations, emitted by seedlings in populations without external GLV supplementation, were too low to accumulate to perceptible amounts. GLVs are predominantly released only after damage, wherefore external GLV concentrations in healthy plant populations are extremely low. Additionally, in the populations without GLV addition we failed to detect any GLVs, whereas for the supplemented boxes we observed high amounts (Figure 17). In contrast to EV, the *irLOX2* monocultures did not respond to GLV supplementation. We suppose that the GLV-lacking *irLOX2* seedlings did not sense GLVs as an effect of silencing *NaLOX2*. To date it is unclear, how plants perceive external GLVs and react to them. It was previously observed that *cis*-3-hexenol supplementation of maize plants resulted in increased expression of LOX (Farag et al., 2005). Whereas these observations did not show induction of the HPL enzyme and its products *cis*-3-hexenal, *trans*-2-hexenal and *cis*-3-hexenol, there was a burst of *cis*-3-hexenyl acetate emission. *Cis*-3-hexenyl acetate is derived from *cis*-3-hexenol through conversion by CoA-Z3H-acetyltransferase. The high emissions of *cis*-3-hexenyl, without synthesis of its precursor *cis*-3-hexenol, evidences that exogenous *cis*-3-hexenol was taken up and converted to the less active acetylated form. In another study it was observed that tomato plants, exposed to infested conspecifics, take up *cis*-3-hexenol and subsequently converts the alcohol to glycosides (Sugimoto et al., 2014). If exposed plants get infested afterwards, attackers have reduced growth and survival rates. Thus, GLV emissions of infested plants are taken up by healthy and intact neighbor plants to develop direct defenses. It is supposed that plants are able to accumulate GLVs in leaves (Matsui, 2006). The uptake could be facilitated through hydrophobic interactions between volatiles and cell wall. Furthermore "...the leaf boundary layer, which is about 5 mm deep, provides a microenvironment that is different from the surrounding area" (Matsui, 2006). Thus, it is thought that GLVs are trapped by plants and accumulate in tissue to noticeable amounts. Based on the observations of Farag (2005) and Sugimoto (2014) we suppose, that trapped GLVs are further converted to molecules, involved in defense response like acetates or glycosides. Whereas glycosides themselves are toxic to attacker, acetate is supposed to prime other defense responses. A study on *Populus* observed that supplementation with *cis*-3-hexenyl acetate and subsequently infestation by gypsy moth larvae resulted in higher concentrations of JA and linolenic acid (Frost et al., 2007). In addition *cis*-3-hexenyl acetate primed expression of genes involved in the oxylipin pathway and direct defense. Through priming plants prepare for possible attack, to elevate or accelerate induced defense responses. It is possible that GLV-

supplemented EV seedlings also developed defense priming in our experiment. This could be tested by infestation of EV monocultures with herbivores after GLV supplementation, to see whether supplemented EV monocultures have better defense than non-supplemented populations. However, by observing internal GLV concentrations of the GLV-supplemented and non-supplemented EV and irLOX2 monocultures we did not find elevated amounts of GLVs in the supplemented plants. This is inconsistent with previous speculations that external GLVs accumulate in plant tissue. Furthermore we did not detect *cis*-3-hexenyl acetate in GLV-supplemented seedlings, which was supposed to be converted in plant tissue from *cis*-3-hexenol. However, it is possible that trapped GLVs were subsequently converted and released from tissue, since Farag (2005) observed emissions of *cis*-3-hexenyl acetate in response to *cis*-3-hexenol supplementation in maize plants. Furthermore the trapped GLVs could also be converted to glycosides, like observed for tomato. But since we did not determine glycosides, we cannot be sure. We suppose that silencing in LOX2 could be paralleled by silencing of LOX2 downstream metabolites, like CoA-Z3H-acetyltransferase. That might explain why irLOX2 plants are insensitive to external GLVs, if they cannot convert them into detectable products. It is possible that silencing in HPL would be accompanied by similar downstream changes due to the resulting decrease in internal GLVs. Thus we predict that, if the insensitivity is due to a downstream product, GLV-deficient irHPL plants also would not respond to external GLVs. If this were not the case, then it would indicate that either a LOX2-specific metabolite, or LOX2 itself were responsible for GLV perception in *N. attenuata*.

Since the GLV supplementations, conducted in our experiment, were similar to GLV blends released from herbivore infested *N. attenuata* plants, we suppose that EV monocultures developed defense response by increasing leaf area. A bigger leaf size makes it less likely that herbivores completely consume seedlings and could also accelerate production of defense metabolites. In leaves, stems and flowers, shoot apical meristems, a group of pluripotent cells, are responsible for development of leaves (McConnell & Barton, 1998). They usually develop in the axils of leaves, where leaves are connected to stems. Destruction of meristems leads to leaf death, since meristems are responsible for development of leaves. But if herbivores fed only on parts of the leaf, the leaf can develop further after herbivory. Thus, plants profit from bigger leaves during infestation and can probably survive, while plants with smaller leaves have a higher risk to die if meristems are damaged.

In addition to observations on LAE, we detected decreased ET emissions in GLV-supplemented EV monocultures. Thus, there seems to be a trade-off between ET synthesis and LAE. That is consistent, since previous investigations observed a trade-off between shade induced growth response and plant defense (McGUIRE & Agrawal, 2005). This trade-off is accompanied by changing sinks of carbon and energy between different metabolic branches (Ronald Pierik, Mommer, & Voesenek, 2013). For instance leaf area and roots are mostly reduced during shade avoidance response

(Kozuka et al., 2010). ET synthesis is important for plants to develop shade avoidance traits, like stem elongation and leaf upward movement, in response to low R/FR. Hence, ET-deficient plants have delayed shade avoidance responses and thus will probably lose competition for light with neighbor plants (Kegge & Pierik, 2010). We assume that increased LAE paralleled by reduced ET emissions in response to external GLVs impairs plants' opportunities to outcompete neighbors. Plants have to set priorities. But since in our experimental set-up plants were very small, they may have only sensed their competitors late in the experiment. In addition, plants exposed to herbivory indicators may have been "focused" on surviving potential attack.

Our set-up had limitations due to which we perhaps failed to observe important cues of defense response or competition. First of all we did not observe the characteristic traits of the shade avoidance syndrome, which is a typical behavior of *N. attenuata* in response to potential competitors. *N. attenuata* plants sense potential competitors through light cues and ET (Kegge & Pierik, 2010). In our seedling populations we had great plant densities of 3555 plants m⁻², and thus we eventually had touching leaves, high ET concentrations and a low R/FR. Low R/FR triggers plants' ET emissions, wherefore canopies with high plant densities accumulate ET to great amounts. As described previously, ET can be sensed by specific negatively regulated receptors that are localized in the cell membrane and in response to high ET concentrations seedlings elongate stems and elevate their leaf angle (R. Pierik, Visser, De Kroon, & Voesenek, 2003). Additionally to ET, plants detect their competitors through light cues, like low R/FR and low blue light, as well as touching leaves, which results in internode, petiole and stem elongation, as well as elevation of leaf angles (Kegge & Pierik, 2010). It was not possible to measure these parameters in our experimental set-up. Furthermore there was not enough space in boxes to observe seedlings over longer periods. Pierik, who observed the shade avoidance syndrome, examined individual seedlings population over a period of about 25 days (Ronald Pierik & de Wit, 2014), whereas our seedlings could only be distinguished for 6 days in the main experiment. During the observations, canopy densities elevated very fast and plants overgrew each other. Thus, it is possible that plants in our set-up were too young to observe an effect of competition in LAE. In conclusion, for further investigations in this set-up, plant density should be reduced by using bigger boxes or fewer seedlings. Furthermore we only focused on aboveground cues, like ET emission, LAE or photosynthetic rate. In our experimental set-up we did not separate roots of the individual seedlings, why we could only determine shoot biomass. It would be interesting to measure the root to shoot ratio, since previous studies observed specifically inhibition of root elongation in Arabidopsis seedlings due to *trans*-2-hexenal supplementation (Mirabella et al., 2008). Furthermore we could not exclude plants belowground root interactions. Plants compete belowground for soil resources like water or nutrients. In dense canopies decrease in nutrients like nitrate, ammonium or phosphate often occurs, and hence competition for these nutrients. As a consequence plants engage in so-called "nutrient foraging" (Ronald Pierik et al., 2007), by stronger biomass allocation to roots which grow into nutrient-rich patches. In addition BVOCs like ET are also emitted by roots and thus are also a belowground cue

of potential competitors. Also belowground competition by allelopathy could not be excluded, where plant roots emit so-called allelochemicals which accumulate in soil and suppresses seed germination, growth and establishment of potential competitors (Kegge & Pierik, 2010). However, the belowground competition traits can be lost when plants have to compete both above- and belowground (Poorter et al., 2012). Thus, it will be better to separate roots of the individual seedlings to exclude belowground interaction and belowground competition traits. Furthermore it is easier to isolate the roots of the individual seedlings to determine root biomass and root to shoot ratio. In order to facilitate root separation, seedlings could be cultured in sand. Finally, we could not observe the whole impact of GLV supplementation, since we did not investigate gene expression of plants and other emissions than ET. This could be done by profiling gene expression. Furthermore we did not observe elevated defense response after GLV supplementation, for instance by metabolism of glycosides. In later investigations one could expose populations subsequently after supplementation to herbivores, in order to investigate whether populations which first sensed GLVs have less damage than other populations.

7. Summary

Plants often compete with each other for light, nutrients, water and space, each genotype trying to maximize its own fitness. Thus, plants have several mechanisms to sense their neighbors, including volatile and light perception. In consequence plants adjust their stem, leaf, petiole, internode and root growth. Furthermore plants can benefit from volatile blends that neighbor plants release. Plants are known to eavesdrop on their neighbors. If neighbor plants are infested by herbivores or pathogens they release specific volatile blends as result of mechanical damage and specific elicitor molecules, e.g. in the insect spit. Eavesdropping plants are forewarned and prime their metabolism to respond to possible infestation. In consequence eavesdropping plants develop stronger defense responses. Important molecules in plant-plant-interaction are *inter alia* green leaf volatiles (GLVs) and the phytohormone ethylene (ET). While ET is known to be involved in sensing close neighbor plants and additionally modify defense response, GLVs are supposed to prime defense response in plants.

In the work reported here we investigated the effects of GLVs and ET in seedling populations, consisting of *Nicotiana attenuata* genotypes which were GLV- and/or ET-deficient, or able to synthesize both. We measured two growth parameters for seedlings: photosynthetic rates and leaf area expansion rates, and as control measurements we quantified seedling and population-level ET emission and internal GLV production of seedlings in different populations. This way we could test the hypotheses that ET and GLVs separately alter the leaf expansion and photosynthetic rates of seedlings in competition, as well as the hypothesis that they have an interactive effect on one or both of these growth-related outcomes.

Furthermore seedling monocultures which either could synthesize GLVs, or were GLV-deficient, were supplemented with a synthetic GLV mix typical of the mix emitted by herbivore-damaged leaves. As a result we observed increased daily LAE in the GLV-producing monocultures, but no effect on the GLV-deficient monocultures. We suggest that seedlings prime themselves for potential herbivory by increasing their leaf area and thus reducing the risk that herbivores destroy the entire seedling shoot including meristems. We suppose that GLV supplementation additionally may lead to elevated direct defense in response to herbivory. Furthermore we observed a trade-off between defense priming by LEA and ET emissions, since ET emissions of GLV-producing monocultures were reduced with external GLV supplementation. Following investigations will determine how metabolism changes due to GLV supplementation and if GLV supplementation primes defense responses. Moreover we want to examine how the trade-off in ET emission and LAE affects seedling competition, since ET is an important cue for neighbor detection.

8. Zusammenfassung

Pflanzen konkurrieren miteinander um Licht, Nährstoffe, Wasser und Raum, um ihre reproduktive Fitness zu erhöhen. Sie besitzen verschiedene Mechanismen, um ihre Nachbarn, beispielsweise durch Duftstoff- oder Lichtempfindung, wahrzunehmen. Im Ergebnis passen Pflanzen ihr Stamm-, Blatt-, Blattstängel-, Internodium- und Wurzelwachstum an die jeweiligen Gegebenheiten an. Des Weiteren können Pflanzen ebenfalls von Duftstoffausströmungen der Nachbarpflanzen profitieren. Pflanzen sind bekannt dafür ihre Nachbarn zu „belauschen“. Wenn diese von Herbivoren oder Pathogenen befallen sind, verströmen sie, ausgelöst durch mechanische Verletzung und bestimmte Molekülen, z.B. im InsektenSpeichel, spezielle Duftstoffzusammensetzungen. Die „belauschenden“ intakten Pflanzen sind somit vorgewarnt und bereiten ihren Metabolismus für einen eventuellen Befall vor. Dadurch können diese später mit stärkerem Abwehrverhalten reagieren. Wichtige Moleküle in der Pflanzeninteraktion sind unter anderem grüne Blattduftstoffe (GLVs), sowie das Phytohormon Etylene (ET). Während ET in der Wahrnehmung naher Pflanzen sowie Modifizierung von Abwehrreaktionen eine wichtige Rolle einnimmt, bereiten GLVs Pflanzen vermutlich auf eine spätere Abwehrreaktion vor.

Im Versuch wurde der Effekt von GLVs und ET in Sämlingspopulationen, bestehend aus *Nicotiana attenuata* Genotypen, welche entweder nicht fähig waren ET und/oder GLVs zu synthetisieren, oder keine Einschränkungen hatten, untersucht. Überprüft wurden zwei Wachstumsparameter für Sämlinge: die Photosyntheserate und die Blattflächenvergrößerung (LAE) der Gesamtpopulationen, sowie die LAE der einzelnen Sämling. Als Kontrolle wurden ET-Emissionen von einzelnen Sämlingen und Populationen sowie interne GLVs der Sämlinge aus verschiedenen Populationen quantifiziert. Auf diesem Wege konnte die Hypothese, dass ET und GLVs jeweils die LAE und Photosyntheserate von Sämlingen in Konkurrenz beeinflussen, nicht bestätigt werden. Auch die Vermutung, dass GLVs und ET einen interaktiven Effekt auf einen oder beide Wachstumsparameter haben, konnte nicht bewiesen werden.

Zusätzlich wurde einigen Sämlingsmonokulturen eine synthetische GLV-Mischung, welche typischerweise nach Insektenbefall emittiert wird, zugesetzt. Die Monokulturen bestanden entweder aus Genotypen, die GLVs synthetisieren konnten oder nicht dazu in der Lage waren. In den GLV synthetisierenden Monokulturen wurde eine erhöhte tägliche LAE beobachtet. Daher wird vermutet, dass Sämlinge sich durch vergrößerte Blattflächen auf mögliche Herbivorie vorbereiten, um zu verhindern, dass Herbivore den gesamten Sämlingspross inklusive der Meristeme zerstören. Wir mutmaßen das GLV-Zugabe ebenfalls zu erhöhter direkter Abwehr nach Insektenbefall führt. Des Weiteren wurde eine Austauschbeziehung zwischen erhöhtem LAE und ET-Emission entdeckt,

da die ET-Emissionen der GLV synthetisierenden Monokulturen unter Zugabe von GLVs vermindert waren. Nachfolgende Versuche sollen zeigen, wie sich der Pflanzenmetabolismus in Folge von GLV-Zugabe verändert und ob GLV-Behandlung schlussendlich zu einer verbesserten Abwehr führt. Des Weiteren soll untersucht werden, wie sich die Austauschbeziehung zwischen ET-Emission und LAE auf den Wettbewerb der Pflanzen untereinander auswirkt, da ET eine wichtige Rolle in der Wahrnehmung naher Pflanzen spielt.

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9. References

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