UNDERSTANDING THE BIOLOGY OF GLANDULAR TRICHOMES AND USE OF EXTERNAL STRESS FOR IMPROVING THE CONTENT OF BIOACTIVE COMPOUNDS IN ARTEMISIA ANNUAL.

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Preface

This thesis has been submitted to Aarhus University as a partial fulfilment of the requirements to obtain the degree of Doctor of Philosophy. My main supervisor was Dr. Martin Jensen, Institute of Food Sciences, Aarhus University, along with co-supervisor Kai Grevsen, Institute of Food Sciences, Aarhus University. The original research was conducted from August 2009 to July 2012, and was primarily based at Institute of Food Sciences in Årslev, Denmark. A research stay was conducted from June to September 2010 at Wageningen University, The Netherlands, at the invitation of Dr. Willemien Lommen, Centre for Crop Systems Analyses.

This thesis is the culmination of 3 years of fruitful work, and has been a journey through many hours of planning and tending experiments, preparing microscopy photos in the darkroom, analysing the photos, analysing results, digging through the reports of piers, and finally the rewarding work of making all ends meet and wrapping them up for publication.

I am indebted to many colleagues, technicians, and administrative staff for their tireless assistance and encouragements. I would particularly like to thank Annette Steen Brandsholm, Karin Henriksen, Birthe Flyger, Jens Barfod, and Astrid Bergman for their assistance with the experiments. A large thank is also due to Dr. Heng Yin for his help and fruitful discussions, and to Dr. Willemien Lommen for inviting me to Wageningen University. Francel Verstappen, Prof. Harro Bouwmeester, Dr. Xavier Fretté, Prof. Lars P. Christensen, and Elise Ivarsen have been highly valued co-authors. My supervisors have been invaluable sparing partners and an inspiration for my progress. Finally, but absolutely not least, I thank my dear family for their patience and love.
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## Abbreviations

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<tr>
<td>AA</td>
<td>Artemisinic acid</td>
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<tr>
<td>AAA</td>
<td>Artemisinic aldehyde</td>
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<tr>
<td>AAOH</td>
<td>Artemisinic alcohol</td>
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<tr>
<td>AN</td>
<td>Artemisinin</td>
</tr>
<tr>
<td>AN-c</td>
<td>Artemisinin related compounds, including artemisinin</td>
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<tr>
<td>COS</td>
<td>Chitosan oligosaccharide</td>
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<tr>
<td>DHAA</td>
<td>Dihydroartemisinic acid</td>
</tr>
<tr>
<td>DHAAA</td>
<td>Dihydroartemisinic aldehyde</td>
</tr>
<tr>
<td>DHAAOH</td>
<td>Dihydroartemisinic alcohol</td>
</tr>
<tr>
<td>DHAAHP</td>
<td>Dihydroartemisinic acid hydrogen peroxide</td>
</tr>
<tr>
<td>EO</td>
<td>Essential oil components</td>
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<td>GT</td>
<td>Glandular trichomes</td>
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<tr>
<td>NGT</td>
<td>Non-glandular trichomes</td>
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<tr>
<td>SA</td>
<td>Salicylic acid</td>
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<tr>
<td>SB</td>
<td>Sandblasting</td>
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<td>SM</td>
<td>Secondary metabolites</td>
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<td>VOC</td>
<td>Volatile organic compounds</td>
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Summary

*Artemisia annua* (*A. annua*) is one of the world’s most important medicinal crops. The plants contain the compound artemisinin (AN), which every year is administered to millions of people against malaria. AN further has the potential to be part of new pharmaceuticals against different viruses, cancer diseases, and infections of bacteria, fungi and protozoa in both humans and animals. The wild type of *A. annua* only contains relatively small concentrations of AN, and enormous research efforts have been invested in improving the AN content and understanding the underlying mechanisms of the biosynthesis of AN. The current project was aimed at creating a deeper understanding of the biology of the glandular trichomes (GT), which produce the AN, and the influence of external stress on factors related to GT and the biosynthesis of AN.

In a series of experiments, large plants of *A. annua* were exposed to different combinations of frequencies and types of stress treatments. Analyses of GT morphology and detailed biochemical analyses of AN-c were performed on leaves sampled at an upper and a lower position in order to observe the differences in response in leaves developed before and after the stress treatments. In one experiment only the upper leaves were sampled, and analysed for differences in AN-c composition and in expression of genes related to the biosynthetic pathway of AN. In another experiment the plants were subjected to different doses of N fertilisation, and differences in GT densities and size parameters were recorded. GT densities and size parameters were analysed by two different methods of fluorescence microscopy, and analyses of AN-c (AN related compounds, including AN) were analysed by UPLC-MRM-MS.

The results showed that, in most plants, the stress treatments did not provoke the upper leaves to initiate more GT and thus create a higher GT density. Only a clonally defined subset of the plants showed slightly, but significantly higher GT densities in response to some treatments. In the lower leaves of an experiment with repeated stress treatments, the GT densities declined significantly in response to one treatment, and showed similar trends in response to the remaining treatments. In an experiment with a single treatment, GT densities of lower leaves were unresponsive to treatments. Size parameters of GT in upper leaves were unresponsive to treatments, whereas lower leaves showed many incidences of significant declines in size parameters, as compared to controls. Detailed biochemical results of AN and its immediate precursors showed that upper leaves did not change neither the concentrations of individual AN-c, the total concentrations of AN-c, nor the ratios between AN-c. In the lower leaves, the concentrations of individual AN-c and the total concentrations of AN-c were lowered non-significantly in response to all stress treatments, whereas ratios between AN-c showed higher proportions of AN and lower proportion of most precursors in response to stress. Correlations were demonstrated between both GT densities, and GT size parameters and, respectively, data on concentrations of several individual AN-c and total concentrations of AN-c. An infection with powdery mildew in a clonally defined subset demonstrated that the higher the degree of mildew infection, the higher the concentration of both AN and total concentration of AN-c in lower leaves. The results of short term analyses of a single round of treatments showed that the upper leaves responded to treatments by the passage of a suggested wave of responses through the pathway of AN. The wave consisted of temporary declines in precursors leading to the formation of dihydroartemisinic acid.
Different stress types apparently produced differently timed passages of the wave of response. These patterns of response waves were not readily reflected in the results of short term gene expression analyses of selected genes. Comparison of the patterns of changes in AN-c compositions and gene expressions in single versus multiple treatments of plants indicated that plants stress treated once were more responsive to treatments than plants treated multiple times. Higher N application to plants caused similar GT densities to plants receiving lower N application, whereas leaf areas and GT sizes increased with higher N application, and thus number of GT per leaf increased with increasing N.

In conclusion, we have demonstrated that, in response to stress, large plants of *A. annua* are less likely to initiate a higher density of GT in newly developed leaves than it has previously been reported for younger plants in the literature. We demonstrated that stress tended to cause decreases in AN concentrations in lower leaves, possibly because stress accelerated the maturation of GT and thus caused increased rates of collapsing GT. We further illustrated that stress was only marginally able to affect the AN related biosynthesis of individual GT, and that the yield of AN and total concentration of AN-c was correlated to the density and size of the GT.
Artemisia annua \( (A. \text{ annua}) \) er en af de vigtigste medicinske afgrøder i verden. Planten indeholder stoffet artemisinin (AN), som er effektivt mod malaria, og som hvert år indgives til millioner af mennesker. AN har desuden potentielle til at blive en del af nye medikamenter mod forskellige virus, kæftsygdomme, og infektioner med bakterier, svampe og protozoer, både i mennesker og i dyr. Vildtypen af \( A. \text{ annua} \) indeholder kun forholdsmæssigt små koncentrationer af AN, og der er lagt en enorm forskningsindsats i at forøge koncentrationen af AN i planterne, og forstå de underliggende mekanismer i biosyntesen af AN. Formålet med det foreliggende projekt var at skabe en dybere biologisk forståelse af de glandulære trikomer (GT), der producerer stoffet AN, og at forstå de underliggende mekanismer i biosyntesen af AN. I en serie eksperimenter blev store \( A. \text{ annua} \) planter eksponeret for forskellige kombinationer af frekvenser og typer af stress behandlinger. Analyser af morfologien af GT og detaljerede biokemiske analyser af AN-c blev udført på bladprøver, som var indsamlet fra henholdsvis en øvre og en nedre position, for derved at kunne observere forskellene i respons i blade, som var udviklet henholdsvis før og efter stress behandlingerne. I et eksperiment indsamlede vi kun de øvre blade, men disse blev analyseret for forskellige sammensætningerne and AN-c og i ekspressionen af gener relatert til biosyntesevejen til AN. I et andet eksperiment blev planterne udsat for forskellige doser af kvælstof gødning, og vi observerede forskellene i GT tætheder og størrelsesparametre. GT tætheder og størrelsesparametre blev analyseret ved hjælp af to forskellige fluorescens mikroskopi metoder og koncentrationer af AN-c (AN relatere molekyler, inklusive AN) blev analyseret ved hjælp af UPLC-MRM-MS.

Resultaterne viste at, for de fleste planters vedkommende, provokerede stress behandlingerne ikke de øvre blade til at initiere flere GT, og medførte dermed ikke en højere GT tæthed. Kun en klondefineret undergruppe af planter viste svagt, men signifikant højere GT tætheder som respons på flere af stress behandlinger. I et eksperiment med gentagte stressbehandlinger faldt GT tætheden på de nedre blade signifikant som respons på en af behandlingerne, og viste tilsvarende tendenser i de resterende stress behandlinger. I et eksperiment med en enkelt stress behandling var GT tæthederne på de nedre blade upåvirkede af stress behandlingerne. Størrelsesparametrene på GT i de øvre blade var upåvirkede af stressbehandlingerne, mens de nedre blade viste mange tilfælde af signifikante fald i størrelserne på GT, sammenlignet med kontrolplanterne. Detaljerede biokemiske resultater af AN og de nærmeste forstadiemolekyler til AN, viste at de øvre blade hverken ændrede koncentrationen af de individuelle AN-c, den totale koncentration af AN-c, eller forholdene mellem AN-c. I de nedre blade blev koncentrationerne af mange af de individuelle AN-c og de totale koncentrationer af AN-c sænket som respons på behandlingerne, mens forholdene mellem AN-c viste højere proportioner af AN og lavere proportioner af de fleste forstadiemolekyler som respons på stress. En infektion af meldug i en klondefineret undergruppe af forsøgsplanterne demonstredede at, des højere grad af infektion, des højere koncentration af AN og total koncentration af AN-c fandtes der i de nedre blade. Analyser over en kort tidsperiode af en enkelt behandlingsrunde viste at de øvre blade responderede på behandlingerne ved passagen af en hypotetisk responsbølge gennem den biosyntetetiske vej mod AN. Responsbølgen bestod af...

Som konklusion har vi demonstreret at store A. annua planter er mindre tilbøjelige til at initiere en højere GT tæthed som respons på stress, end det tidligere er blevet påvist på mindre planter i litteraturen. Desuden demonstrerede vi, at stress havde en tendens til at forårsage lavere AN koncentrationer i de nedre blade, muligvis fordi stress behandlingerne opaccelererede modningsprocesserne i GT, og dermed forårsagede forhøjede frekvenser af GT der kollapsede. Desuden blev det vist at stress kun marginalt var i stand til at indvirke på biosyntesen af AN i det enkelte GT, og at udbyttet af AN og total AN-c var korreleret til antallet og størrelsen af GT.
Chapter 1: Introduction to thesis

1. Introduction to thesis

The present study was part of a multi-institutional project aimed at introducing *A. annua* as a crop in Denmark, and at developing a production of feedstuff containing antiprotozoal and antibacterial bioactive fractions of *A. annua* for poultry production. The part of the project, which I am responsible for, was providing background knowledge, which can be used to implement cultivation methods improving the biochemical composition of *A. annua*. In order to reach this level, an understanding of the underlying mechanisms and dynamics is needed. Thus, the execution of experiments of this study was aimed at testing fundamental scientific hypotheses and creating a frame of knowledge about the GT of *A. annua* and the biosynthesis of AN in stress treated plants. At the same time it was prioritized to approximate the execution of the experiments as much as possible to an actual crop situation. This primarily meant that our experimental plants were of large stature and were either grown in the field or in a semi-protected greenhouse.

The present project was operating in the difficult cross field between the very different scientific disciplines of histology, horticulture, biochemistry, genetics, plant physiology, etc. A major challenge has been to select and understand the publications most relevant to the present project among the immense published knowledge on *A. annua* and AN. To set things in perspective, searching for “*Artemisia annua*” and “artemisinin” in “Web of Knowledge” gives approx. 2300 and 5000 hits, respectively, and performing the same search in Google gives approx. 500,000 and 1,000,000 hits, respectively. To narrow the scope and complexity of this thesis, I have chosen mainly to present knowledge on the secondary metabolites related to the biosynthesis of AN, and thus not go into detail with the numerous other secondary metabolites (SM) found in *A. annua*.

The objective of this thesis was to improve the understanding of the biology of GT and the use of external stress for improving the content of bioactive compounds in *A. annua*. The thesis is composed of four chapters of literature review, (Chapters 2-5), in which I introduce some background knowledge on *A. annua*, GT, AN, and stress. Part of this knowledge is essential for the understanding of the subsequent experiments and discussions, and other parts of the information are primarily included to illustrate the importance of research in improving the content of AN in cultivated *A. annua* plants. The main hypotheses of the project are presented (Chapter 6). Subsequently I have included 5 papers (Chapters 7-11), which are either published or intended for publication in peer reviewed journals. Papers I and III are published. Paper II is submitted. Paper IV is presented as a manuscript close to submission, and Paper V is presented as a poster, which is intended to be written up as a publication. The papers are themselves closed entities, but as the experiments and the writing of the papers provided a progressing accumulation of knowledge and speculations, I have chosen to elaborate on these discussions and on the perspectives in relation to cultivation practices and future research in a separate discussion (Chapter 12). Finally I will sum it all up in a conclusion (Chapter 13).
Chapter 2: Artemisia annua

2. Artemisia annua

2.1. Botany, distribution and natural biology

Artemisia annua \((A. annua)\) belongs to the family Asteraceae of the order Asterales, and the genus Artemisia, which contains several hundred species. The members of the family are characterised by small composite flowers, and by often having strong aromas and bitter tastes from terpenoids and sesquiterpene lactones. \(A. annua\) is, most likely, native to habitats of lower mountainous areas of Northern China (Ferreira et al., 1997). Due to human intervention it is now naturalised in many parts of the world, and particularly subtropical to temperate climatic conditions suits \(A. annua\) well. The native environment of \(A. annua\) is in a steppe vegetation, and thus it particularly thrives under semiarid conditions (Ferreira et al., 1997). \(A. annua\) is intolerant to frost exceeding a few degrees frosts (Personal observation). The growth form of \(A. annua\) is a shrub like herb, which under optimal conditions can attain heights of 3-4 meters. The lifecycle of \(A. annua\) is annual, and the initiation of flowering is governed by day length, with photoperiods of less than 12 hours over a prolonged period initiating flowering. In tropical regions, homogenous day lengths often initiates flowering at an early developmental stage of the plants, whereas artificially supplying photoperiods of more than 12 hours delays the flower initiation and the plants can consequently remain in the vegetative stage for several years. In northerly temperate regions (i.e. Denmark), the difference between the timing of the initiation of flowering and the onset of frost is usually too short for the plants to be able to complete the production of viable seeds.

2.2. Human cultivation.

Chinese herbalists have collected and semi-cultivated \(A. annua\) in the wild for millennia, but nowadays an increasing need for raw materials has demanded a more systematic cultivation of the plant. Since the 1970’s, the production of \(A. annua\) has largely been based on collections from the wild and on small scale farmers growing the plant as a supplementary cash crop along with their normal crops. Although recent years has seen the establishment of more and more production sites dedicated to the cultivation of \(A. annua\), a substantial part of the worldwide production of \(A. annua\) is still based on the input from small scale farmers. The production time of \(A. annua\) is relatively long (up to 8-9 month), as compared to other cash crops selectable for the local farmers. Their produce is collected by local merchants, and often goes through a chain of traders before it reaches the extraction sites. Plummeting world market prices in 2008-9 discouraged many small scale farmers continual production. However, considerable loss of production (up to 30%) due to bad weather conditions in China and Vietnam in 2011 (Vietnam, 2011) caused an alarming scarcity of artemisinin (AN), and prices have consequently increased considerably again. The dependability on the willingness of small scale farmers to gamble on the production of \(A. annua\), combined with world traders manipulating prices by withholding final produces, has made the annual production and predictability of future production difficult. Initiatives to promote stability in annual production, producer prices, and end user prices have been made at national, NGO, and WHO levels, and has, among other things, resulted in the formation of the primarily UN funded A2S2 (Assured Artemisinin Supply System), which include providing an economical safety net under farmers and
Chapter 2: Artemisia annua

extractors, and encouraging the establishment of long term production contracts (Madagascar, 2010).

Exact estimates of worldwide annual production of artemisinin and areas occupied by the farming of *A. annua* have historically been difficult to obtain, but in recent years, international symposia, and a closer contact between national and international institutions has apparently created a clearer overview. From 2003-2006 artemisinin production increased from app. 10 ton per year to app. 160 ton per year and the production area increased from 2,000 hectares to 25,000 hectares (Kindermans et al., 2007). The years 2008 and 2009 were poor production years, and AN production was down to app. 110 ton per year, rising to 140 ton in 2010, and a projected 160 ton in 2011 (A2S2, 2011). The worldwide demand in 2012 has been estimated to be between 160 and 180 ton (A2S2, 2011; Vietnam, 2011). Approximately 70% of the worldwide production of AN are supplied by China, 10% by Vietnam, 10% by countries in East Africa, and the rest by other countries (York, 2009; Vietnam, 2011).

2.3. Medicinal uses of Artemisia annua

*A. annua* plants have been used for medicinal purposes by Chinese herbalists for millennia. A Chinese pharmacologist recommended the use of *Artemisia* against fever in the 4. th century, and in the 16. th century, *A. annua* was recommended against malaria (Ferreira et al., 1997). In the late 1960’s Chinese researchers evaluated the effectiveness of a number of traditional herbs including *A. annua* against malaria, and demonstrated that extracts of *A. annua* in organic solvents showed activity against the protozoan parasites *Plasmodium vivax* and *P. falciparum*, causing malaria in humans. In 1972, the main active compound, AN was isolated and structurally defined by Chinese researchers, and the research in *A. annua* soon became worldwide (Ferreira et al., 1997; Brown, 2010). In the following decades it has been further confirmed that AN is indeed the active compound in *A. annua*, and that it has an antimalarial effect (e.g. Klayman et al., 1984; Klayman, 1985). This was later verified in large clinical trials (SEAQUAMAT, 2005), which, in 2006, led WHO (World Health Organisation) to include intravenous artesunate (a derivative of AN) as the recommended first-line treatment for severe malaria (Maude et al., 2010). Since then, the production of *A. annua* and medicaments containing AN derived compounds has grown explosively. Malaria is considered to be the most serious infectious disease in the world, with at least 300 million cases and 2 million deaths annually (Brown, 2010), so the arrival of a new antimalarial remedy was greeted very welcome. However, great concerns were voiced about the growing problem with *Plasmodium* parasites becoming tolerant to traditional AN therapies, and that this could possibly similarly happen with AN derived compounds. To prevent the risk of tolerance, WHO has recommended that AN derived compounds are never to be used alone (Artemisinin Mono Therapy, AMT), but is always administered together with at least one other antimalarial agent (Artemisinin Combination Therapy, ACT), which is usually one or more quinine based compounds (Maude et al., 2010). Several reports have documented a growing problem with *Plasmodium* parasites becoming resistant/tolerant to AN and its derivatives in an area at the Thai-Cambodian border, where AN has been administered for centuries (Dondorp et al., 2009; Soomro et al., 2011; Cheeseman et al., 2012). The exact resistance mechanism is currently unknown, but has been demonstrated to be facilitated by a single gene mutation in infected mice, and is believed to be facilitated by a similarly simple mutation mechanism in human malaria.
parasites (Cheeseman et al., 2012). The use of ACT has greatly improved the probability of long term effect of AN derived compounds (Dondorp et al., 2009).

The fact that the AN molecule is highly insoluble in water presents a number of challenges with the use of AN as a pharmaceutical. The administration of AN to the target patients (human or animal) is complicated both at the level of absorption into the body, in the bodily internal transport of AN to the intended place of activity, and because AN undergoes a relatively rapid clearance within in the body (Lapenna et al., 2009). Weathers et al., (2011) reported that when A. annua leaves were prepared in tea and administered to humans, only about 2% of the available AN reappeared in the bloodstream, and when administered to mice as dried leaves, about 0.4% reappeared in the bloodstream. Though Ferreira et al. (2010) suggested that the administration of tea or dried plants material, has the advantage that particularly some of the accompanying antioxidant phenolics in A. annua may have positive synergic effects with AN, most of the treatment of malaria by is presently performed by the administration of semi-synthesised derivatives of AN.

The AN is extracted from A. annua plant material, and is sold on the world market in its crystallised form. In specialised pharmaceutical facilities, the refined AN (Figure 1A) is reduced to dihydroartemisinin (Figure 1B) and can be administered to malaria patients successfully in this form (Soomro et al., 2011). But low stability of the compound, has led to the development of a series of further derivations including artesunate, artemether and arteether (Figure 1C-E). Artesunate has proven to be the most stable compound, both in storage and in the body of patients, and has become the most widely used formulation in the world (Brown, 2010).

Figure 1. Artemisinin and close derivatives. A - Artemisinin, B - Dihydroartemisinin, C - Artemether, D - Arteether, and E - Artesunate. Modified from Brown (2010) and Soomro et al. (2011).

2.4. Bioactivity of artemisinin and its derivatives

AN has a wide range of pharmaceutical applications, most prominently against malaria, but is also active against several other protozoal and schistosomal infections, cancer tumours, vira and fungi (Soomro et al. 2011). The feature making the AN molecule efficient as a drug is the endoperoxide bridge in the molecule (Figure 1A, Thesis). Only a few other natural products are reported to possess a peroxide bridge, including ascaridole from the Chilean Peumus boldus (Opsenica et al., 2007). The bridge is composed of two oxygen molecules, which are relatively strongly bound to the molecule, but under the right circumstances the bridge can be broken and free radicals are exposed. The exact
mechanism of the breaking of the peroxide bridge by the *Plasmodium* parasites in infected human blood cells is under debate, and at least two models are in play (O’Neill et al., 2010). In the most commonly accepted “Reductive scission model”, the *Plasmodium* parasites degrade the host haemoglobin through a series of steps within the food vacuoles of the parasite into ions with low valency such as ferrous heme. These bind to artemisinin (or its derivatives) and induce a reductive environment, which opens the peroxide bridge and thereby produce oxygen centred radicals, which subsequently rearrange into carbon centred free radicals (O’Neill et al., 2010). Free radicals are highly reactive and cause havoc in their immediate surroundings, so when released inside the parasites, these are killed. The stability of peroxides in contact with intact oxyhemoglobin (most abundant form of iron in humans), and reactivity with low valent heme may explain the selective toxicity of AN against infected, but not healthy blood cells (O’Neill et al., 2010). The anticancer effect is believed to be connected to the understanding that tumour cells maintain a high intracellular iron concentration to sustain continued cell division (Tin et al., 2012). The primary effect of the subsequently released free radicals is proposed to be induction of oxidative DNA damage to the cancer cells, which subsequently either die, cease to multiply, are inhibited in their hormone production, are inhibited in migrating in the host body, or are otherwise inhibited in causing damage to the host (Beekman et al., 1996; Berdelle et al., 2011; Crespo-Ortiz, et al., 2012; Efferth et al., 2011; Tin et al., 2012; Mao et al., 2012). The exact mechanism of the activity of AN and its derivatives against vira and fungi is less understood, but several experiments have demonstrated strong effects (Efferth et al., 2011; Chou et al., 2011; Galal et al., 2005; Gautam et al., 2011; Tang et al., 2000). Of particular interest to the present project is the possible effect of AN and other essential oil components (EO) from *A. annua* on different poultry diseases such as necrotic enteritis, coccidiosis and black head. These effects are currently being investigated in a larger research project, which we take part in (Engberg et al., 2012).

AN and its derivatives are suited as pharmaceuticals because they are so well tolerated by humans and other vertebrate animals and has shown very few side effects (Dondorp et al., 2009; Maude et al., 2010). This is believed to be connected to the presumption that AN and derivative molecules only become toxic when they encounter a highly reductive environment, which opens the peroxide bridge and creates the toxic free radicals. The presence of a highly reductive environment is relatively rare in normally functioning bodies, and accidental release of free radicals is quickly countered by the release of anti-oxidants (Efferth, 2007).
3. Trichomes

3.1. Introduction to trichomes

Plant trichomes have been defined as protuberances with a morphogenetic origin in epidermal cells, and which have relatively well defined size parameters (Werker, 2000; Wagner et al., 2004). Trichomes can have a multitude of appearances, and can roughly be separated in two groups, non-glandular (NGT) and glandular trichomes (GT). NGT are characterized by not having any storage capacity, but apart from that, no common feature can characterize the diversity of the structure. NGT can appear as hairs, hooks, needles, plates, “umbrellas”, etc. and have very diverse functions, including prevention of desiccation or soaking, assistance to seed dispersal, cold- and heat insulation, herbivore deterrence, etc. Probably the most important NGT to humans are the hair-like trichomes on the seeds of cotton (Levin, 1973; Wagner et al., 2004). GT, on the other hand, have the ability to store, and sometimes synthesize SM and sequester different compounds, which are unwanted in the plants in secluded organs. 20-30% of all vascular plants invest considerable resources in building, maintaining and filling GT on the aerial surfaces of the plants (Levin, 1973; Wagner, 1991; Ågren and Schemske, 1993). GT may have several secondary functions to the plants, but in the present paper focus will primarily be on the stress protective functionality.

3.2. Glandular trichomes in Artemisia annua

Though A. annua possess hair-like NGT, these are only peripherally dealt with here. In relation to the production of AN and other SM, the GT are much more interesting, and here receives the main focus. The GT of A. annua are composed of 10 cells combined into 5 cell pairs, and therefore termed biseriate (Ferreira and Jannick, 1995). In A. annua, GT are primarily thought to contribute to the stress protection (Hu et al., 1993; Duke and Paul, 1993; Duke, 1994). Comparative studies between a glanded and a glandless biotype of A. annua showed that the glanded cultivar contained a higher cumulative amount of SM than the glandless, and that the SM production in the glandless biotype was restricted to a few sesquiterpene compounds, not including AN (Duke et al., 1994; Tellez et al., 1999). This indicates that the GT are the primary, but not the sole site of the overall production of SM. Delicate laser dissection studies of GT of A. annua (Olsson et al., 2009; Olofsson et al., 2012) showed that key enzymes of AN production were exclusively expressed in the GT. This makes the understanding of GT essential for the overall understanding of SM production in A. annua.

3.3. Initiation and ontogeny of glandular trichomes

The understanding of how and especially when the initiation of GT takes place during the development of the leaves is critical for the present project, as part of the aim was to investigate the possibility of influencing the number of GT by applying external stress. It is thus important to know if it is possible to initiate new GT in existing leaves, or if it is only possible to influence the initiation of GT on leaves initiated later in the development of the plant. Despite the fact that the initiation of GT creates the starting point for all further production of AN in A. annua, this subject has been dedicated surprisingly little scientific focus.
In the 1970’s, 80’s and 90’s a wave of microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) studies and reviews elucidated many aspects of GT of plants in general (Werker, 2000; Duke et al., 2000), in the genus Artemisia as a whole (Kelsey and Shafizadeh, 1980; A. nova; Corsi and Nencioni, 1995; A. nitida); Ascensão and Pais, 1987 (A. campestris); Werker et al., 1994 (A. dracunculus)), and in A. annua in particular (Duke and Paul, 1993; Hu et al., 1993; Duke et al., 1994; Ferreira and Jannick, 1995).

A consensus was created that the ontogeny of GT in the genus Artemisia is acropetal, and begins with the enlargement of a single epidermal cell, which, after considerable expansion above the leaf plane, divides anticlinally. The two resulting cells divide periclinally to form two new cells, which in turn divide periclinally. This process is continued until the GT consists of five cell pairs (Duke and Paul, 1993). This phase in the development of the GT has been termed presecretive (Werker, 2000). The initiation of GT in plants in general has been stated to commence at an early stage of leaf development often prior to stomatal development, sometimes even before the leaf primordium can be distinguished (Werker, 2000). This was confirmed for A. annua by Duke and Paul (1993) and for A. campestris by Ascensão and Pais (1987), who also stated that the final number of GT in the leaves was defined at an early developmental stage of the leaves. Consequently the current consensus is that the number of GT of the fully developed leaf in A. annua is predetermined already at an early developmental stage of the leaves. Since this wave of microscopy studies, the direct study of the initiation and ontogeny of GT in A. annua has been very limited, and the interest in GT has moved on to other levels of information.

3.4. Morphology, development and maturation of glandular trichomes

The two lower cell pairs primarily function as stalk cells, carrying two sub-apical cell pairs, which in turn carry the apical cell pair (Duke et al., 1994). The stalk cells contain chloroplast and the organelle structure resembles normal epidermal cell structure. The subapical cells contain modified chloroplasts, and the apical cells contain no chloroplasts, but possess reticulated ingrowth in the cell walls, probably to facilitate SM export. All GT cells contain high amounts of smooth endoplasmatic reticulum, which is associated with synthesis of lipids, including oils, phospholipids and steroids, and metabolism of carbohydrates. Particularly the areas of reticulated ingrowth in the cell wall of the apical cells contain high amounts of endoplasmatic reticulum (Duke and Paul, 1993). Shortly after the differentiation of the 10 cells, the GT go into the secreting phase (Werker, 2000), and the 6 apical and subapical cells (secreting cells) start synthesising SM and secrete these into a subcuticular sac, which only borders with the secreting cells and not the stalk cells (Duke and Paul, 1993). The subcuticular sac is gradually filled with SM, and the sac separates from the cell walls of the secreting cells, forming a bilobed sac (Duke and Paul, 1993). The cuticular detachment is considered to be associated with the termination of secretory activity (Ferreira et al., 1997). Only few researchers have investigated the speed by which the subcuticular sac is filled with SM, though Duke and Paul (1993) stated that it was “rapid”. To our knowledge no reports have been published on the possible difference in timing of the secretion of the numerous different SM.

The GT are proposed to go through a process of maturation and later of senescence, and thus enter a postsecretive phase (Ascensão and Pais, 1987; Werker, 2000). Duke and Paul
(1993) reported that during the maturation process, the amount of endoplasmatic reticulum in the secreting cells increases, until a point where cell membranes begin to break, resulting in loss of organellar and cellular integrity of secretive cells. In other species of GT bearing plants, the stalk cells are shown to become more active, and are suggested to contribute to the terpene catabolism (Werker, 2000). The exact progress of the maturation and timing of the senescence of GT in A. annua is still unresolved.

3.5. Excretion of secondary metabolites

Anyone who has been near an A. annua plant will have noticed the strong odour of volatiles emanating from the plant, but the intent observer will notice that younger plants left undisturbed do not smell until touched, whereas older plants have a constant “background” smell, which can be intensified by touching the plants. This is due to the mechanism of excretion of SM from the plant. Stored SM in GT of plants in general can be released to the surroundings by either a continuous leaking of SM, or by a single event of rupturing the storage organ and thus releasing all the stored SM. The SM of A. annua are by many thought only to be released by the rupture of GT (Duke and Paul, 1993; Ferreira et al. 1997; Lommen et al., 2006; Zhang et al., 2006; Lommen et al., 2007; Arsenault et al., 2010; Kjaer et al., 2012a). This can happen either by forceful breaking of the subcuticular sac (i.e. by rubbing young leaves), or by auto-collapse of mature and senesced GT.

The mechanisms of maturation and auto-collapse of individual GT is difficult to investigate, as it would essentially require following the development of the exact same GT throughout their lifetime, and presently no research has focused on setting up such an experiment. But research has provided some indications of the functionality of the mechanism. Duke and Paul (1993) reported that the subcuticular sac would start splitting in the central area of the bilobed structure. Zhang et al. (2006) demonstrated that 4% of GT in upper leaves were split open, 12% were split in middle leaves, and 25% were split in lower leaves of large A. annua plants. Lommen et al. (2006) followed the development of several sets of comparable leaves, and demonstrated a gradual decline in detectable GT after the leaves reached a certain age, coinciding with a maximum in the size of the leaves. Arsenault et al. (2010) found that the number of GT in relation to epidermal cells was at a maximum when leaves reached full size and decreased thereafter. Ferreira et al. (1997) stated that intact GT were observed in inflorescences during their development, but seldom after anthesis. This “disappearance” of GT possibly indicates that the GT collapses with leaf age.

The understanding of the mechanisms of excretion of SM from the GT and collapse of GT are of importance to the present project, because stress treatments were hypothesised to affect the maturation rate, both in terms of synthesis of SM and ultimately the collapse of GT (Section 12.2., Thesis).
Chapter 4: Artemisinin

4. Artemisinin

4.1. Introduction

AN is a highly oxygenated sesquiterpene lactone consisting of three isoprene units. It contains the unique 1,2,4-trioxane ring structure, where the peroxide bridge (Figure 1A) is particularly interesting, because this is responsible for the primary pharmaceutical properties of the compound (Brown, 2010). The pure compound crystalizes, and is highly insoluble in water, but readily soluble in organic solvents.

AN belongs to the terpenes, which are found in almost all life forms, and is, by mass, the most abundant class of small-molecule natural products on earth, and approximately 65,000 different terpenes are identified (Oldfield and Lin, 2012). Terpenes are constituted of one or several C5 isoprene “building blocks”, and the isoprenes dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IPP) are the most common structure motifs in plants (Weathers et al., 2006). The cyclic condensation of the terpenes are either completed in the cytosol by the mevalonate pathway (MVA) or in the plastids by the methylenerythritol phosphate pathway (MEP) or in a combination of both (Weathers et al., 2006; Towler and Weathers, 2007). *A. annua* synthesises around 50 different sesquiterpenes, and within the plants, a continuous competition for structure motifs exists among the pathways leading to the different terpenes (Brown, 2010). The outcome of the competition is determined by a combination of genetically and environmentally determined enzyme expressions, spatial separation of the location of pathways, and different inhibition mechanisms (Olsson et al., 2009). The more advanced the process of cyclic condensation is, the narrower is the competition from the different pathways. For AN, a critical starting point is amorpha-4,11-diene, which is dedicated to the AN pathway. Based on knowledge from Towler and Weathers (2007), Olsson et al. (2009), Wang et al. (2009), and their own data obtained from radio labelled CO2, Schramek et al. (2010) presented an intricate mechanism of a gradually advancing spatial separation of the process leading to AN. They proposed that an isopentenyl diphosphate unit (MEP pathway in plastids) and a isoprenes dimethylallyl diphosphate unit (MVA pathway in cytosol) are synthesised in the green subapical cells of the GT (section 3.4.), and are then transported to the non green apical cells where they are converted to amorpha-4,11-diene, and thus trapping the precursor of the AN pathway in the apical cells. Though intriguing, this proposal has later been hampered by Olofsson et al. (2012) finding that key enzymes in the AN pathway are also found in the subapical cells. Clearly more studies are needed to fully understand the subcellular and cellular compartmentation of AN synthesis in the GT.

4.2. Biosynthetic pathway of artemisinin

The current understanding of the biosynthetic pathway of AN has many parents (Schmid and Hofheinz, 1983; Klayman et al., 1984; Bouwmeester et al., 1999; Wallaart et al., 1999; Sy and Brown, 2002; Bertea et al., 2005; Covello et al., 2007; Towler and Weathers, 2007; Covello, 2008; Ollsson et al., 2009; Teoh et al., Arsenault et al., 2010; Brown, 2010; Polichuk et al., 2010; Schramek et al., 2010; Maes et al., 2011; Oldfield and Lin, 2012; Olofsson et al., 2012). Most of the pathway seems to be well described, though diverging opinions still exist in some parts of pathway. Some of these disagreements may very well be attributed to the existence of at least two different chemotypes of *A. annua* (Wallaart et al., 2001) (Section 4.3., Thesis).
In the following, numbers in brackets refer to process numbers in Figure 2. The dedicated pathway towards AN begins with the cyclisation of farnesyl diphosphate to form amorpha-4,11-diene by amorphadiene synthase (ADS) (1) (Wallaart et al., 2001). Artemisinic alcohol (AAOH) is formed by oxidation by cytochrome P450 (CYP71AV1) (2) (Ro et al., 2006; Teoh et al., 2006). From here the exact pathway to AN and other end products is not clearly demonstrated. Covello et al. (2007) proposed a possible pathway from AAOH to
dihydroartemisinic alcohol (DHAAOH) (3), but this has not been mentioned by any other subsequent reports, and remains unresolved. Thus, probably only artemisinic aldehyde (AAA) is formed from AAOH by a further oxidation by CYP71AV1 (4) (Ro et al., 2006; Teoh et al., 2006). The important cross road between the artemisinic acid (AA) and the dihydroartemisinic acid (DHAA) pathways seems to be at AAA (Zhang et al., 2008; Rýden et al., 2010), and not as suggested at the AAOH or AA levels (Bertea et al., 2005). From here AAA can be reduced by the double bond artemisinic aldehyde reductase (DBR2) (Zhang et al., 2008) to form dihydroartemisinic aldehyde (DHAAA) (6). DHAAA then enters in a competition between the enzymes dihydroartemisinic aldehyde reductase (RED1) (Rýden et al., 2010) and aldehyde dehydrogenase (ALDH1) (Polichuk et al., 2010) (8). If RED1 wins out, it reduces the DHAAA to dihydroartemisinic alcohol (DHAAOH) (5), whereas it does not convert the closely similar AAA (Rýden et al., 2010). It has been suggested that DHAAOH is a dead end in the pathway, though the functionality of the plants producing enzymes which divert and disrupts the pathway towards AN has been questioned (Rýden et al., 2010). It has been suggested that RED1 has a larger energy barrier to overcome than the ALDH1 (8), and thus functions as an “overflow valve” to rid the cells of some of the potentially toxic DHAA if ALDH1 cannot keep up with the clearance of the DHAAA (8) (Rýden et al., 2010). The existence of a process of reverting the DHAAOH to DHAAA by oxidation by CYP71AV1 or ALDH1 (5) has been hypothesised, but has presently not been demonstrated (Rýden et al., 2010; Maes et al., 2011). If, however, the direct pathway towards AN is followed and ALDH1 wins the competition with RED1, DHAAA is oxidised by ALDH1 to DHAA (8), which is one of the major storage precursors to AN. The further conversion of DHAA to AN runs in two non enzymatic steps (Wallaart et al., 2001; Sy and Brown, 2002). First DHAA passes an oxidation energy barrier by energy input from light to form dihydroartemisinic acid hydroperoxide (DHAAHP) (10), which in the presence of oxygen forms the characteristic endoperoxide bridge in AN (12). DHAAHP is a highly unstable molecule, and few reports have demonstrated in planta quantification of DHAAHP (Wallaart et al., 1999), but researchers have hypothesised that DHAAHP can temporarily exist in substantial quantities in the GT (Lommen et al., 2006; Lommen et al., 2007; Paper IV, Thesis).

If, however, the other path of the cross road at AAA wins out, AAA can be oxidised by either CYP71AV1 or ALDH1 (7) (Nguyen et al., 2011) to form AA, which through two steps of non enzymatic photo and air oxidations can form arteannuin B (AB) (9) and subsequently artemisitene (AT) (11) (Teoh et al., 2007). The molecules of this branch of the pathway after AAA seemingly has no known possibility to enter any pathway leading to AN, but throughout the history of the unravelling of the biosynthetic pathways of AN, almost all researchers have left a backdoor open for this possibility. This could happen either by a conversion of AT to AN (13), or by a reversion of AA to AAA (reverse (7)), which would then go through DHAAA (6) on the path to AN. Brown and Sy (2007) and Brown (2010) introduced radioactively labelled metabolites at different levels of the pathway both into in vitro, in vivo, and in planta systems and established that AA has no direct path to AN. However, the findings have not been verified for all chemotypes, and are as such not conclusive for all A. annua plants.

A major factor determining the fate of AAA at the cross road described above, seems to be the genetic background of the plant material. Wallaart et al. (2001) presented two
different chemotypes of *A. annua*, Type I, containing relatively high levels of AN and DHAA and low levels of AA, and Type II, containing relatively high levels of AA and low levels of AN and DHAA. Wang et al. (2009) described metabolic differences between the types and Maes et al. (2011) and Wu et al. (2011) demonstrated that a range of phytohormones initiated two different chemotypic expression patterns of key genes involved in the biosynthetic pathway of AN. The *A. annua* plants used in our experiments (cv. Artemis, F2 seeds, Mediplant, Switzerland), were estimated to be of chemotype I, because the samples contained high levels of AN and DHAA as compared to levels of AA (see results of Papers I, II, and IV, Thesis).

### 4.3. Genes of *Artemisia annua*

Along the way of determining the enzymes responsible for the different steps in the metabolic pathway of AN, the primer sequences for the expression of the enzymes have been determined. Some enzymes are well know from other organisms (*ALDH1* and *ADS*) and others are only known from *A. annua*, *RED1* (Ryden et al., 2010) *CYP71AV1* (Ro et al., 2006; Teoh et al., 2006), and *DBR2* (Zhang et al., 2008). Other genes affecting the artemisinin biosynthetic pathway have been identified. For example, a *WRKY* transcription factor, *AaWRKY1*, has been suggested to regulate the expression of the *ADS* gene (Ma et al., 2009). Two putative transcript factors “transparenta testa glabra 1” (*TTG1*) and “enhancer of glabra3” (*GL3*), which promote trichome initiation were isolated from an *A. annua* subtractive cDNA library (Liu et al., 2009). Graham et al. (2010) furthermore performed an extensive genetic survey by various genetic marker methods of 242 crossings of *A. annua* var. ‘Artemis’, and found a number of markers associated with high yields of AN, and thus facilitated the identification of high-yielding cultivars for future breeding purposes.
Chapter 5: Protection and stress

5. Protection and stress

5.1. Introduction to stress and protection mechanisms

The stress of plants is generally understood as the reaction of the system of a plant to extreme environmental factors that, depending on their intensity and duration, may cause significant changes in the system (Heil, 2010). There is a diversity of types of stress that may affect plants. Biotic stress is stress that occurs as a result of disadvantageous conditions of plants caused by other living organisms such as bacteria, viruses, fungi, parasites, predators, and other plants. Abiotic stress may be of physical or chemical character, and include the negative impact of non-living factors such as intense sunlight or wind that may cause harm to the plants, edaphic factors, water status, nutrient status, etc. (Heil, 2010).

Plants are stationary, as opposed to most animals, and thus cannot avoid danger by escaping, but need to take up the fight where they are (Gutbrodt et al., 2012). Therefore plants have evolved a multitude of different and often intricate strategies to protect themselves (Kessler and Baldwin, 2002). Protection strategies against biotic stresses can broadly be divided into 3 categories; - direct protection, where the plants deter the danger by bad smell, irritants, thorns, poisons etc.; - indirect protection, where the plants invite “bodyguards” i.e. ants, or mimic the appearance (visual or smell) of another plant known to be dangerous by predators, etc.; - or tolerance, where the plants simply live with the danger of occasionally losing parts of their system or members to predators or diseases (Kessler and Baldwin, 2002). The reason why plants utilising the last strategy may have survived natural selection, is that any protection mechanism has a price for the plants, and thus evolution may have favoured individuals, which have saved their resources to the occasional calamity of a passing predator or disease, and not spent the resources on protection mechanisms (Kessler and Baldwin, 2002). The price of building and maintaining a protection mechanism can be substantial, and in heavily armed plants such as some cactuses, half of the aboveground mass is constituted of inert thorns, not contributing to the metabolism of the plant. Plants relying on chemical protection systems also pay a price for the investment in the synthesis of compounds, the building of storage facilities, and sometimes even in the wellbeing of the plant itself, as some chemical protection molecules (or their intermediates) exert harmful or disadvantageous effects to the plants (Heil, 2010). This has led to the development of different mechanisms of plasticity in protection expression, where the plants only initiate protection mechanisms when and where they are necessary (Heil, 2010), as opposed to e.g. thorns, which are a permanent installation.

Phenotypic plasticity is the ability of a plant to express different phenotypes in response to environmental conditions (Heil, 2010). A factor which is particularly difficult to anticipate is the presence of enemies in terms of predators or diseases, and many plants have evolved strategies of having a basic reservoir of chemical protection molecules, which can quickly be enhanced when the plants are stressed. The enhancement can happen at a local level, where only the particular attacked organ (leaf, branch, etc.) is responding, on a systemic level, where all of the stressed plant responds, or on a population-wide level, where the stressed plant alarms neighbouring plant by emitting volatile organic compounds (VOC) (Mur et al., 1997; Mattiacci et al., 2001; Heil and Ton,
In these escalating response strategies there is an inbuilt risk of overspending on the up regulation of the protection status. As the initial stress, which set of the alarm, may have been caused by a passing danger. Thus, the organ, plant, or even the whole population of plants may have invested in defence molecules, which no longer have an enemy. This problem is reduced in many plants by a second layer of plasticity in the alarm responses of plants, namely priming. Primed tissues initially show no or little enhanced level of response, but when the alarm is set of again, the plants respond with a high level of response (Heil, 2010). The understanding of plasticity in protection against stress is in the core of the present project, as the main scope was the possibility of affecting the protection strategies of the plants against abiotic and the mimicking of biotic stress.

5.2. Protection against stress in Artemisia annua

Though the essence of why A. annua is interesting as a crop lies in their protection molecules, no single published report has, to our knowledge, centred its focus on what the plants are protecting themselves from. Countless reports are listing and quantifying the content of SM, and testify that A. annua produces a relatively high number (almost 600) and high quantities of molecules with a potential protective function, as compared to other plants (Brown et al., 2010). Similarly, countless reports demonstrate that the plants do respond to different direct stress or simulated stress (Table 1, Thesis). But the overall question of why the plants have evolved protection mechanisms is seldom asked.

Hu et al., (1993) reported that A. annua are rarely attacked by herbivores, and in our experience most vertebrate and invertebrate animals tend to avoid direct contact with the plants, and no bite marks on leaves were ever observed. In the experimental fields at Wageningen University, The Netherlands, plants of A. annua were bitten by rabbits when very young, but not later in growth (Dr. Willemien Lommen, pers. comm.). In our experiments the bioavailable SM of A. annua apparently did not exclude the growth of several unidentified fungi, including a powdery mildew (Golovinomyces sp.). Duke et al. (1987) reported that some constituents of A. annua are phytotoxic, possibly exerting an allelopathic function on plants competing for the same resources. Jessing et al. (2009) demonstrated that the presence of composted dead A. annua plant material in the soil had a deterring effect on several earth dwelling animals including earthworms, and thus exerting a possible “cleaning-effect” of the earth for the next generation of seeds. Insecticide experiments have shown high efficiency of both crude extract of A. annua and pure AN (Maggi et al. 2005). But an overall understanding of the enemies of A. annua remains to be presented.

A. annua apparently relies solely on a chemical protection strategy, as it has no external structures such as thorns etc. to deter herbivores, and the hair-like trichomes do not appear to act in the protection of the plants. Chemical protection may consist of deterring from a distance by smell or irritants, deterring close-up by touch-provoked smell or irritant release, and deterring by ingestion of bad tasting or poisonous compounds (Levin, 1973; Wagner, 1991). A. annua utilize all three strategies. Most of the chemical protection compounds in the aerial parts of A. annua are produced and stored in GT (Brown, 2010). Here they stay until the GT rupture and release the SM. In young, undisturbed plants this happens only when the plants are touched, whereas in lower parts of older plants it
happens continuously, and particularly when touched. This provides the plants with a cover of smell, which deters predators from a distance and particularly if they get close enough to touch the plants. If they start eating the plants, they get a bitter taste and possibly poisonous effects from AN and other compounds.

Observing the plants during growth gives some clues to how the plants prioritise their protection throughout the development. In the first weeks after the plants have emerged from the seeds, the leaves have very few GT (Personal observation) and only have a very weak bitter taste, indicating that the plants are allocating most resources to growth rather than protection. Later in the development the density of GT in newly developed leaves becomes increasingly higher, and the bitter taste is strongly intensified. *A. annua* seems to hold a particular interest in protecting the inflorescences, as these are densely covered with faster maturing GT, which rupture and release their content during anthesis to the point where almost all GT have ruptured at the end of anthesis (Duke and Paul, 1993) and the content of AN and flavonoids declined (Baraldi et al., 2008).

Whether *A. annua* exhibits a locally or a systemically induced protection response remains largely unresolved. Almost all the stress experiments (including ours) discussed in Papers I-IV and elsewhere in this thesis have been conducted by treating the entire plants and analysing the result on a whole-plant scale or on local levels of the plants, and not as e.g. Holeski et al. (2007) who pinched one set of leaves of *Mimulus guttatus* and observed an induced response in later developed leaves. The only exception found was Rai et al. (2011) who pre-treated plant with UV-B and -C radiation, planted them in the field, and observed significant increases in AN content at full bloom, as compared to controls. Though treatments also resulted in shorter plants, and thus a possible up-concentration of AN, this might indicate systemic acquired stress response.

Experiments to elucidate the possible mechanism of priming of *A. annua* plants to maintain a lower threshold of initiating the response to attacks has not yet been demonstrated in the literature, as it has in other plants (Heil, 2010; Massoud et al., 2012; Winter et al., 2012). Neither has the existence of a possible epigenetic heritability of a systemic acquired response to the seeds of the next generation been investigated or demonstrated, as it has in e.g. *Arabidopsis* and tomato plants (Slaughte et al., 2011; Luna et al., 2012). A possible population-wide alarm system by VOC has neither been investigated in *A. annua*. However, in other species of *Artemisia* (*A. tridentate*, *A. cana*, and *A. douglasiana*) experiments have elucidated that the emissions of VOC play key roles in the defence response to wounding and the subsequent damage induced by herbivores, often to the extent that the emission of VOC play a larger role than the internal signal transport within the individual plants (Karban et al., 2006; Kessler et al., 2006; Shiojiri and Karban, 2006; Shiojiri and Karban, 2008; Shiojiri et al. 2009). Species of *Artemisia* are reportedly very active emitters of a broad spectrum of VOC, including methyl jasmonate, terpenoids and a range of green leaf VOC (Kessler et al., 2006), but the exact mechanisms of interplant communication remain uncertain, though methyl jasmonate seems to play a key role (Preston et al., 2001; Preston et al., 2002; Preston et al., 2004). Rapparini et al. (2008) investigated the effect of arbuscular mycorrhizal colonisation on the emission of VOC from *A. annua* plants, and found only few insignificant effects. The results of our experiments do have communication by VOC as a possible explanation to otherwise unexplained differences in the observed composition.
of compounds between untreated control plants in two experiments where the neighbouring plant were treated a single time or multiple times (Paper IV and Section 12.5. Thesis). Clearly there is an open field for further investigations in these three mechanisms.

5.3. Signal control of protection mechanisms

The part of the plants that first senses a stress factor is a receptor. Once a signal is picked up by a receptor, the signals are transmitted intercellularly and activate nuclear transcription to initiate the effects of a certain set of genes. These activated genes allow the plant to respond to the stress it is experiencing (Heil, 2010). When a plant experiences a stress event, the first step in a cascade of events is the release of one or a number of bursts of reactive oxygen species (ROS), which are chemically reactive molecules containing oxygen. ROS are potentially toxic to the cells, but through evolution, intricate methods have evolved to limit the harmful effects by selective ROS scavenging, and to contain the ROS in certain cellular or systemic organelles. This has made it possible to utilise ROS as practical signal transmitters (Mittler et al., 2011). The signal is transmitted by a wave of auto-propagation, as each cell experiencing a ROS burst from its neighbouring cell will automatically (if other critical circumstances are present) activate its own ROS producing mechanisms, and thus the signal spreads as rings in the water (Mittler et al., 2011). The practicality lies in the speed by which ROS are produced and released (and degraded when the use is ended), and the speed by which a signal can travel through the plant (up to 8.4 cm min⁻¹ has been reported in Arabidopsis) (Mittler et al., 2011). The ROS wave is even suggested to set off an electric signal along plasma membranes similar to nerve signals in higher animals (Mittler et al., 2011). But one wave alone can only send the message “Alarm!”, so how do the attacked cells convey more specific messages? This is apparently highly controversial and under current investigation (Mittler et al., 2011; Kreslavski et al., 2012; Suzuki et al., 2012). Several scenarios have been presented, including the sending of several ROS waves oscillating in specific amplitude and frequency patterns, and thus transmitting a coded signal. Another scenario suggests that the specificity in the signal lies in accompanying signals of other effectors (hormones, peptides, lipids etc.). Mittler et al. (2011) states that the specificity probably lies in a combination of the two scenarios mentioned above and other parallel signalling pathways, such as Ca²⁺ dependent pathways.

After broadcasting the signals, the next step in the response cascade after an event of stress, is that the correct site in the plant receives and decodes the signal and initiates the correct response. This would, in the case of A. annua, be in the GT. As the transmission of signals remains unclear, the understanding of the reception and decoding of the signal remains similarly unclear. But Kreslavski et al. (2012) hypothesised that the ROS sensors may be transcription factors or protein kinases, which are able to change their activity in response to the appearance of ROS signals. The activation of transcription factors may influence the transcriptions of genes directly involved in the response to stress or the transcriptions of genes coding for intermediary signal molecules, e.g. phytohormones (Kreslavski et al., 2012). Phytohormones particularly associated with metabolic protection mechanisms in plants are salicylic acid and jasmonates, but also gibberellic acid, abscisic acid and cytokinins are associated with protection related cell and organelle adaptations, e.g. GT development (Nguyen et al., 2011).
A number of transcription factors which affects the biosynthesis of AN have been identified in *A. annua*. Ma et al. (2009) isolated the factor *AaWRKY7*, which was found to regulate the expression of the *ADS* gene, a proposal which was supported by results of Maes et al.(2011). Yu et al. (2012) isolated the two transcription factors *AaERF1* and *AaERF1*, which were shown to induce promotor activity of *ADS* and *CYP71AV1*. Liu et al. (2009) isolated *TTG1* and *GL3*, which are thought to promote GT initiation. All the above factors have shown positive associations to the jasmonate group of phytohormones (Nguyen et al., 2011). It has long been hypothesised that *A. annua* produces and uses jasmonates in or in close connection to the GT, but not until recently has this been backed up by evidence, as Lu et al. (2012) cloned the *A. annua* version of the *AOS* gene (*AaAOS*), which facilitates the first committed step in the biosynthetic pathway of jasmonate. They did not directly pinpoint the production of jasmonates to happen in the GT, but nevertheless showed that the more GT rich the plant tissue was, the higher was the transcription of *AaAOS* (Lu et al., 2012).

### 5.4. Agents of stress responses

A large number of compounds and physical conditions can act to raise the protection level in plants. These can roughly be divided into signal molecules, effectors, elicitors, chemicals, wounding, and growth related conditions. Signal molecules are a large class of molecules, including the plant hormones Jasmonic acid (JA), salicylic acid (SA), gibberellic acid (*GA$_3$*), abscisic acid (ABA), which have specific or generalistic functions in the effectuation of a stress response. Of these, JA and SA are the molecules most often associated with defence responses (Bent and Mackey, 2007). Effectors are molecules (often small molecules), which have the ability to bind to proteins (e.g. enzymes) and alter their bioactivity, either by activation or inhibition (Maldonado-Calderón et al., 2012). Effector activity can e.g. be increased by the presence of elicitors. Elicitors are molecules (lipids, carbohydrates or proteins) which the plants recognise as part of an attacking organism, and thus initiate a specific or a broad stress response (Maldonado-Calderón et al., 2012). Chemicals may be toxic to the plant in some way, and often initiate a broad stress response (Bent and Mackey, 2007). Wounding of a plant is a serious event, and the plant initiates a cascade of ROS wave initiated stress responses (Liu et al., 2010). Physical conditions, such as water stress, nutrient deficiency/surplus, etc. can furthermore act to raise the defence related stress level (Marchese et al., 2010). In table 1, I have listed the majority of the studies utilising stress agents to elucidate the effect of stress on the protection level of *A. annua* plants, and the effect on AN-c, GT, and gene expression is summarized.
Table 1. Summary of the majority of experiments using stress treatments on A. annua plants. Results are often approximated from graphs, and should be read with reservations. The presented results are often only the most extreme results of the individual experiments, and do as such not do justice to the meticulous work of these authors.

<table>
<thead>
<tr>
<th>Author</th>
<th>Plant material</th>
<th>Type</th>
<th>Treatments</th>
<th>AN related response</th>
<th>GT related response</th>
<th>Gene related response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aftab et al. (2010a)</td>
<td>Field, 90 days old plant, entire plant harvested</td>
<td>?</td>
<td>Spraying multiple times with salicylic acid</td>
<td>AN 1.3 fold up</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Aftab et al. (2010b)</td>
<td>Field trial, 90 and 120 days</td>
<td>?</td>
<td>Treatments with irradiated sodium alginate</td>
<td>AN 1.2 fold up, max. in flowering plant</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Aftab et al. (2011a)</td>
<td>Wild type, growth chamber, 45 days</td>
<td>II (?)</td>
<td>Spraying with jasmonic acid</td>
<td>AN temp. up at 24h and again at 14d</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Aftab et al. (2011b)</td>
<td>Wild collected, 40 cm tall</td>
<td>?</td>
<td>Spraying with Chitosan oligosaccharide</td>
<td>AN 1.4 fold up at intermittent B concentration</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Barpaei et al. (2011)</td>
<td>Field, 150 days</td>
<td>?</td>
<td>Boron (B) supply</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Davies et al. (2009)</td>
<td>Pots outside, app. 6 weeks</td>
<td>I (?)</td>
<td>Nitrogen (N) and potassium (K) supply</td>
<td>AN decreasing with higher N and unchanged with higher K</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Davies et al. (2012)</td>
<td>Pots outside, app. 6 weeks</td>
<td>I (?)</td>
<td>Phosphorus (P) and Boron (B) supply</td>
<td>AN unchanged with higher P and increasing to a certain level with higher B</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ferreira (2007)</td>
<td>Greenhouse, 60 days old</td>
<td>I</td>
<td>Nutrient deficiency, Nitrogen (N), Phosphorus (P), potassium (K)</td>
<td>Deficiency of N and P gave app. unchanged AN, DHAA and AA. Deficiency of K gave higher AN, and unchanged DHAA and AA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Guo et al. (2010)</td>
<td>Chamber cultured to flowering, 30 days old</td>
<td>?</td>
<td>Soaking roots in salicylic acid</td>
<td>AN 2-fold up</td>
<td>NA</td>
<td>Up regulation of ADS (24h)</td>
</tr>
<tr>
<td>Guo et al. (2010)</td>
<td>Chamber cultured to flowering, 30 days old</td>
<td>?</td>
<td>Soaking roots in methyl jasmonate</td>
<td>AN 1.3-fold up</td>
<td>NA</td>
<td>Up regulation of ADS (24h)</td>
</tr>
<tr>
<td>Heng et al. (2011) (Paper III, Thesis)</td>
<td>Greenhouse, 13 weeks</td>
<td>I</td>
<td>Spraying with salicylic acid and chitosan oligosaccharide a single time</td>
<td>AN slightly up after up 48h</td>
<td>NA</td>
<td>ADS up, CPR slightly up, CYP71AC1 unchanged, TG1 up or unchanged, AaWRKY1 unchanged (24-48h)</td>
</tr>
<tr>
<td>Kapoor et al. (2007)</td>
<td>Field, 35 days</td>
<td>I (?)</td>
<td>Soaking roots in methyl jasmonate</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Kjaer et al. (2012a,b) (Papers I and II, Thesis)</td>
<td>Greenhouse, 9 weeks</td>
<td>I</td>
<td>Sandblasting and spraying with salicylic acid, NaCl, H2O2, and chitosan oligosaccharide multiple times</td>
<td>AN slightly down after 5 weeks, DHAA slightly down after 5 weeks, AA slightly down after 5 weeks, AAA slightly down after 5 weeks, DHAAOH slightly down after 5 weeks</td>
<td>GT density slightly up or unchanged and GT area slightly down or unchanged in upper leaves. GT density slightly down or unchanged and GT area slightly down or unchanged in lower leaves,</td>
<td>NA</td>
</tr>
<tr>
<td>Kjaer et al. (2012c) (Paper IV, Thesis)</td>
<td>Greenhouse, 13 weeks</td>
<td>I</td>
<td>Sandblasting and spraying with salicylic acid, NaCl, H2O2, and chitosan oligosaccharide multiple times</td>
<td>AN slightly down after 168h, DHAA temporarily sign. down, AAA temporarily sign. down, DHAAOH temporarily sign. down</td>
<td>NA</td>
<td>ADS up, CPR mainly unchanged, CYP71AC1 up or unchanged, TG1 up, unchanged or down, AaWRKY1 up or unchanged (24-166h)</td>
</tr>
<tr>
<td>Lei et al. (2011)</td>
<td>Greenhouse, clones, 45 days</td>
<td>I (?)</td>
<td>Spraying with Chitosan oligosaccharide</td>
<td>AN 1.7 fold up after 24h</td>
<td>NA</td>
<td>Short term up regulation of ISPH, DXR, DMR2, HMGR, FDS, FSP, CYP71AV1, ADS with different timings</td>
</tr>
<tr>
<td>Liu et al. (2009)</td>
<td>Field, close to flowering</td>
<td>?</td>
<td>Spraying with jasmonic acid</td>
<td>AN 1.7 fold up at 14 days</td>
<td>GT density 2 fold up at 14 days</td>
<td>Up regulation of TTG1</td>
</tr>
<tr>
<td>Liu et al. (2010)</td>
<td>Field trial, 90 and 120 days</td>
<td>?</td>
<td>Treatments with irradiated sodium alginate</td>
<td>AN 1.2 fold up, max. in flowering plant</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Chapter 5: Protection and stress
<table>
<thead>
<tr>
<th>Author</th>
<th>Plant material</th>
<th>Type</th>
<th>Treatments</th>
<th>AN related response</th>
<th>GT related response</th>
<th>Gene related response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannan et al. (2010)</td>
<td>Shoot cultures</td>
<td>Spraying and soaking with jasmonate for 2 weeks</td>
<td>AN 2.5 fold up after 5 weeks</td>
<td>Density 1.6 fold up, area unchanged</td>
<td>FDS up, DHR2 up, ADS up, ALDH1 down, CYP71AV1 up, CPR up (2 weeks)</td>
<td>Up regulation of CYP71AV1, no change in ADS</td>
</tr>
<tr>
<td>Marchese et al. (2010)</td>
<td>Growth chamber, 67 days</td>
<td>I (?): Water deficit</td>
<td>AN higher at 38h, but not 66h</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pu et al. (2009)</td>
<td>Greenhouse, 40 days old</td>
<td>I (?): Spraying with salicylic acid</td>
<td>AN 1.8 fold up after 96h</td>
<td>Density 4.5 fold up, area 1.5 fold up</td>
<td>FDS - , DHR2 - , ADS up, ALDH1 up, CYP71AV1 up, CPR up (2 weeks)</td>
<td>NA</td>
</tr>
<tr>
<td>Rai et al. (2011a)</td>
<td>Wild collected, Hydroponic, 50 days</td>
<td>? : Arsenic treatments</td>
<td>AN 1.5 fold up after 7 days</td>
<td>GT more turgid than control</td>
<td>Up regulation of HMG1R, FDS, CYP71AV1, ADS</td>
<td>Up regulation of HMG1R, FDS, CYP71AV1, CPR, ADS, unchanged GPPS, RPS in both UV-B and UV-C</td>
</tr>
<tr>
<td>Rai et al. (2011b)</td>
<td>Wild collected seeds, 45, 75, and 95 days</td>
<td>? : UV radiation before transplantations</td>
<td>AN at preflowering in leaves 1.8 fold up (UV-B and UV-C) AN at full bloom unchanged (UV-B and UV-C)</td>
<td>Density 2.4 fold up, area 1.8 fold down</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Wang et al. (2007)</td>
<td>Field trial, 15-100 days</td>
<td>? : Different degrees of shade of sunlight</td>
<td>AN 1.4 fold higher in 100% irr. than 15% irr.</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Wang et al. (2010)</td>
<td>Chamber cultured, 50 days old</td>
<td>I (?): Spraying with methyl jasmonate</td>
<td>AN 1.5 fold up after 8 days</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Wu et al. (2011)</td>
<td>Growth chamber, 8 leafed young seed plants</td>
<td>I : Spraying with methyl jasmonate</td>
<td>AN 4.5 fold up after 24h</td>
<td>NA</td>
<td>Up regulation of ADS, CYP71AV1, DHR2, ALDH1, no change in CPR (24h)</td>
<td>Down regulation of ADS, CYP71AV1, DBR2, CPR, up regulation of ALDH1 (24h)</td>
</tr>
<tr>
<td></td>
<td>II : Spraying with methyl jasmonate</td>
<td></td>
<td>AN 1.2 fold up after 24h</td>
<td>NA</td>
<td>Down regulation of DHR2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DHAA not detectable after 24h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA not detectable after 24h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA 7.8 fold up after 24h</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6. Introduction to experiments

In the light of the contemporary knowledge of the effect of stress on the protection mechanisms of *A. annua*, we set up a series of experiments to further strengthen the understanding. We prioritised to approximate the experiments to a crop situation, and thus only utilised large plants cultivated in the field or in a greenhouse under natural sunlight. When we planned our stress experiments, we aimed at applying as broad a spectrum of stress agents as possible. Thus, we applied the plant hormones JA and SA, as these had already been demonstrated to elicit an effect on the defence responses. To elicit a ROS like response, we sprayed with \( \text{H}_2\text{O}_2 \), though this has not previously been reported to have been applied to *A. annua*. To elicit the recognition of an attacking organism, we sprayed with chitosan oligosaccharide. To mimic the attack of a sucking or biting predator we applied two types of wounding. And to elicit a response to an environmental chemical stress agent, we sprayed with NaCl solutions, though this has not previously been reported to have been applied to *A. annua*. We set up the following hypotheses:

- Externally applied stress will increase the density of GT in leaves developed after the stress treatments.
- Externally applied stress will not change the density of GT in leaves developed before the stress treatments.
- Externally applied stress will increase the size of GT in both leaves developed before and after the stress treatments.
- Externally applied stress will cause an elevated individual and total concentration of AN-c.
- Externally applied stress will change the ratio between concentrations of AN-c.
- Density of GT will be positively related to individual and total concentrations of AN-c.
- Density of GT will be related to ratios between concentrations of AN-c.
- Size parameters of GT will be positively related to individual and total concentrations of AN-c.
- Size parameters of GT will be related to ratios between concentrations of AN-c.
- Externally applied stress will, over time, change the concentration of individual and cumulative AN-c.
- Externally applied stress will, over time, change the expression of genes related to the AN biosynthetic pathway.
- Different nitrogen applications will cause different GT densities.
- Different nitrogen applications will cause different GT size parameters.

The hypotheses are discussed and validated in the following papers (Chapters 7-11) and in Chapters 12 and 13 of this thesis.
Chapter 7: Paper I

7. PAPER I

PUBLISHED

“Effect of external stress on density and size of glandular trichomes in fully grown Artemisia annua L., the source of anti-malarial artemisinin”

Anders Kjær, Kai Grevsen, Martin Jensen

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Effect of external stress on density and size of glandular trichomes in full-grown *Artemisia annua*, the source of anti-malarial artemisinin

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Abstract

Background and aims

Glandular trichomes (GT) of *Artemisia annua* produce valuable compounds for pharmaceutical and industrial uses, most notably the anti-malarial artemisinin. Our aim was to find out whether the density, number and size of GT can be manipulated to advantage by environmental stress. A range of external stress treatments, including stress response regulators, was therefore given to fully grown plants under field and greenhouse conditions.

Methodology

In a field experiment (Ex1), seed-grown plants were subjected to chemical or physical stress and plants analysed after 5 weeks. In a greenhouse experiment (Ex2), three groups of clonally derived plants were stressed at weekly intervals for 5 weeks. Stress treatments included sandblasting, leaf cutting and spraying with jasmonic acid, salicylic acid, chitosan oligosaccharide (COS), H$_2$O$_2$ (HP) and NaCl (SC) at different concentrations. Leaves from an upper and a lower position on the plants were analysed by fluorescence microscopy to determine the density and size of GT.

Principal results

Densities of GT on upper leaves of full-grown *A. annua* plants generally showed no response to external stress and only plants from one clone of Ex2 supported the hypothesis that increased density of GT was inducible in upper leaves by stress (significant for SC, HP and COS). The density of GT on lower leaves was not affected by stress in any experiment. Glandular trichomes were significantly smaller on the lower leaves in response to stress in Ex2, and a similar non-significant trend was observed in Ex1.

Conclusions

The results indicate a dynamic system in which stress treatments of large *A. annua* plants had a minor promoting effect on the initiation of GT in developing leaves, and a maturing effect of GT later in the lifetime of the individual GT. The hypothesis that applying stress can induce larger GT or more numerous GT was rejected.

Introduction

Glandular trichomes (GT) on *Artemisia annua* produce and store the anti-malarial compound artemisinin (AN) and other secondary metabolites (SM) that have several pharmaceutical and industrial uses. The present paper investigates the spatial and temporal distribution...

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of GT on leaves and tests the hypotheses that environmental stress influences the size and density of GT.

Many vascular plants invest considerable resources in building, maintaining and filling GT on aerial surfaces (Levin 1973; Wagner 1991; Aagren and Schemanske 1993). Glandular trichomes have several secondary functions and in A. annua GT are thought to contribute to plant defence (Duke and Paul 1993; Hu et al. 1993; Duke 1994). The biseriate capitulate GT consist of 10 cells stacked in pairs (Duke and Paul 1993). The four lower cells function primarily as a stalk for the six topmost cells. These six cells all border the secretory cavity and contribute to filling the apical subcuticular space with SM. A laser dissection study of GT of A. annua (Olsson et al. 2009) showed that key enzymes of AN production were expressed exclusively in the two apical cells of GT. Studies on the initiation and development of GT in the genus Artemisia have established that differentiation of foliar cells into GT cells is completed in a very young primordial stage of the leaf (Duke and Paul 1993—in A. annua; Ascensão and Pais 1987—in A. canepetris). As shown by Duke and Paul (1993) and discussed by Davies et al. (2009), the number of GT of the fully developed leaf in A. annua is predetermined at this early primordial stage.

Werker (2000) defined GT as mature when surrounding cells senesce and stop secreting SM to the central cavity. Glandular trichomes of some species continuously excrete SM, while in other species excretion of all the SM occurred during a single destructive rupture of the GT. In A. annua, Duke and Paul (1993) reported that the subcuticular sac was visibly broken in many mature glands, and they considered A. annua to be among the species that release SM by rupturing of the GT. Both Lommen et al. (2006—A. annua) and Shanker et al. (1999—Mentha arvensis) followed the development and found that GT densities were highest at the maximum size of leaves, after which time densities decreased rapidly, suggesting that some GT ruptured over time. Similarly, Arsenault et al. (2010—A. annua) found that the number of GT in relation to epidermal cells was optimal as leaves reached full size and decreased thereafter. Ferreiro and Janick (1995) focused on the floral morphology of A. annua and found that physiological maturity of GT in the inflorescence coincided with full bloom.

If stress is defined as external conditions that are suboptimal for growth, past work has demonstrated that stress can affect the formation of GT on leaves. For example, Solanum lycopersicum (Solanaceae) treated with methyl jasmonate developed nine times higher GT densities compared with untreated controls (Boughton et al. 2005). Similarly, jasmonic acid (JA), gibberellic acid and benzylaminopurine (BAP, a synthetic cytokinin) applied to Arabidopsis thaliana (Brassicaceae) resulted in up to four times higher GT densities (Maes et al. 2008). In the same species, wounding and JA also significantly increased the number of GT, whereas salicylic acid (SA) decreased the number of GT (Traw and Bergelson 2003). In Mimulus guttatus (Scrophulariaceae), damage on leaves that appeared early in the season increased GT formation on leaves that developed later. It was also shown that this trait was maternally transmitted to progeny (Holeski 2007). Medicago sativa (Asteraceae) subjected to leaf wounding and/or water shortage has been shown to produce increased GT density (Gonzalez et al. 2008). Within A. annua, few published experiments are available linking external stress and the density and size of GT. Liersch et al. (1986) concluded that the growth regulators diaminozide and chlormequat may influence formation of GT, and Kapoor et al. (2007) demonstrated that arbuscular mycorrhizal fungi and added micronutrients increased GT densities, while Liu et al. (2009) showed that application of JA increased GT densities. Maes et al. (2011) demonstrated that the application of JA and gibberellin acid, but not cytokinin (BAP), increased the density of GT. They further determined that a cultivar with a low AN content exhibited a greater plasticity for change in the GT density than a cultivar with higher AN content. This finding suggested a mechanism whereby this species can regulate its capacity for GT on the leaves. Arsenault et al. (2010) determined that foliar GT distribution differed when plants were vegetative or reproductive and found a strong positive relationship between AN content and GT densities, regardless of leaf type. This strong relationship between GT densities, AN and key precursors was confirmed by Graham et al. (2010). Lommen et al. (2006) have given extensive insights into the differing densities of GT on individual leaves during development and maturation. Nguyen et al. (2011) reviewed comprehensively the influence of external factors on the production of GT and AN in A. annua.

Our treatments were selected to represent a broad range of stress-inducing agents. Mechanical damage was applied by sandblasting (SB; Ex1 and Ex2) or cutting the leaves (Ex1). Indirect damage resulting from the osmotic stress was induced by spraying with NaCl (SC; Ex2). Mimicking stress conditions by triggering the recognition of molecules associated with insect and fungus attacks (Zheng et al. 2010; Lei et al. 2011) was achieved by applying chitosan oligosaccharide (COS; Ex1 and Ex2). Treatments with H2O2 (HP; Ex2) mimicked the bursts of reactive oxygen species triggered by the plants under stress (Neill et al. 2002; Mittler et al. 2011). The hormones JA (Ex1) and SA (Ex1 and Ex2)
were applied since they are directly involved in the internal stress management of the plants (Parthier 1990; Wasternack 2007; Pu et al. 2009).

These treatments helped test two primary hypotheses: firstly, that stress treatments on pre-primordial leaves can increase the density and size of GT; secondly, that stress treatments applied to mature leaves are capable of altering GT density and size. These hypotheses were tested on both seed-grown and clonally propagated A. annua in the field and greenhouse.

Materials and methods

Plant material Ex1

Seeds of A. annua (cv. Artemis, F2 seeds; Mediplant, Switzerland) were sown in a greenhouse, and 6 weeks later (June 2009) planted in the field at Research Station Aarslev, Denmark (55°18’N, 10°27’E). The soil was a sandy loam, fertilized with 100 kg N ha⁻¹ prior to planting. No artificial irrigation was provided and weeding was manual. Plants were 18 weeks old and 90–110 cm in height at the onset of treatments, and 150–180 cm tall at sampling.

Plant material Ex2

From a field population of seed-propagated A. annua (cv. Artemis, F2 seeds; Mediplant), three plants were randomly selected as mother plants for clonal propagation by cuttings and sufficient plants were obtained during two rounds of multiplication carried out in a greenhouse. Tip cuttings were ~10 cm long, comprising 4–5 internodes longer than 1 cm. Cuttings were rooted after 2–3 weeks, and potted in 3.5-L containers with Pindstrup no. 2 peat moss (Pindstrup Mosebrug, Denmark). Plants were transferred from the nursery to the experimental greenhouse, and allowed to acclimatize for 7 days before the onset of treatments. Plants were 9 weeks from propagation and 80–110 cm tall at the onset of the treatments, and 150–190 cm tall at sampling. During the experiment, plants were drip irrigated twice diurnally with a liquid fertilizer adapted for Asteraceae.

Experimental design Ex1

The experiment was carried out during September–October 2009. A randomized complete block design with subsampling with three blocks was set up in a field. The 12 different treatments were randomly represented within each block. Beds with three rows of plants were subdivided into plots containing 18 plants, which all received the same treatment. Three random plants from each plot were sampled for the present experiment. Plants were spaced 50 cm apart and individual beds were separated by 150 cm of bare soil. With a manual garden vapourizer (Gardena), JA (J2500; Sigma Aldrich) solutions (95% water: 5% ethanol) and SA (S7401; Sigma Aldrich) solutions (100% water) were sprayed in aliquots of 10 mL per plant at concentrations of 0.05, 0.5 and 5.0 mM. Chitosan oligosaccharide (provided by Dalian Glycobio Ltd, China) was sprayed in aliquots of 10 mL per plant in a concentration of 200 mg L⁻¹. Sandblasting was carried out using a small hobby sandblaster (Badger, model 260-3, aluminium oxide particles), and leaf cutting was carried out with hand-held scissors (see Table 1 for an overview of treatments). Chemical treatments and SB were focused exclusively on the top section of the main shoot including the lower leaf (Fig. 1) by placing a plastic container around the shoot while spraying/SB. Leaf cutting consisted of systematic removal of parts of the leaves on all side branches above the lower leaf marking. Treatments were carried out in September 2009 and repeated on half of the sandblasted and leaf-cut plots once each week for a 5-week time period. Treatments caused no physical changes to the plants, except for the scissor cuts and tiny brown spots caused by SB. The upper leaf was defined as the first leaf below the apex on the main stem with internodes longer than 2 cm (Fig. 1). To recognize leaves that were at a comparable developmental stage at the onset of the experiment, the main stem below the 10th internode from the upper leaf was marked on all the plants at the onset of treatments. The leaf above this mark was the lower leaf. On 29 September 2009, the lower leaves and the upper leaves were sampled along with the two leaves above and below the respective leaves. Leaves were immediately placed in plastic bags between filter papers, soaked in ice-cold water and stored at 3 °C. To investigate differences in morphology of adaxial and abaxial leaf sides and to follow the GT development in time, leaves from nine untreated plants were sampled once a week during the experiment from leaf positions as described above.

Experimental design Ex2

The experiment was carried out during April–May 2010. A randomized complete block experimental design with subsampling was set up in a greenhouse divided into two self-contained compartments. In each compartment, three beds of plants were established, each bed consisting of 3 × 23 plants of one of the three clones. Plants were 50 cm apart and individual beds were separated by 100 cm of gravel. Every second row of three plants was left as a guard row, allowing for 11 treatable subplots of three plants in each bed (2 blocks × 3 clones × 3 plants = 18 subplots per treatment). Treatments were performed weekly for 5 weeks, and included NaCl (Salina) at 1 and 10 g L⁻¹ aqueous solutions, SA
Table 1: All treatments of the field experiment (Ex1) and greenhouse experiment (Ex2) with the abbreviations used in the text and figures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Abbreviation</th>
<th>Cumulative treatment per plant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Field (Ex1)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>Salicylic acid 0.05 mM</td>
<td>SA1</td>
<td>0.07 mg</td>
</tr>
<tr>
<td>Salicylic acid 0.50 mM</td>
<td>SA2</td>
<td>0.69 mg</td>
</tr>
<tr>
<td>Salicylic acid 5.00 mM</td>
<td>SA3</td>
<td>6.91 mg</td>
</tr>
<tr>
<td>Jasmonic acid 0.05 mM</td>
<td>JA1</td>
<td>0.11 mg</td>
</tr>
<tr>
<td>Jasmonic acid 0.50 mM</td>
<td>JA2</td>
<td>1.05 mg</td>
</tr>
<tr>
<td>Jasmonic acid 5.00 mM</td>
<td>JA3</td>
<td>10.51 mg</td>
</tr>
<tr>
<td>Chitosan oligosaccharide&lt;sup&gt;a&lt;/sup&gt; 0.2 g L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>COS</td>
<td>20.00 mg</td>
</tr>
<tr>
<td>Sandblasting × 1</td>
<td>SB × 1</td>
<td>× 1</td>
</tr>
<tr>
<td>Sandblasting × 4</td>
<td>SB × 4</td>
<td>× 4</td>
</tr>
<tr>
<td>Cutting × 1</td>
<td>Cut × 1</td>
<td>× 1</td>
</tr>
<tr>
<td>Cutting × 4</td>
<td>Cut × 4</td>
<td>× 4</td>
</tr>
<tr>
<td><strong>Greenhouse (Ex2)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>NaCl 1.0 g L&lt;sup&gt;-1&lt;/sup&gt;−17.11 mM</td>
<td>SC 1</td>
<td>140.00 mg</td>
</tr>
<tr>
<td>NaCl 10.0 g L&lt;sup&gt;-1&lt;/sup&gt;−171.12 mM</td>
<td>SC 2</td>
<td>140.00 mg</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; 0.1 %−29.37 mM</td>
<td>HP 1</td>
<td>0.14 mL&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; 1.0 %−293.71 mM</td>
<td>HP 2</td>
<td>1.40 mL&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Salicylic acid 0.1 g L&lt;sup&gt;-1&lt;/sup&gt;−0.72 mM</td>
<td>SA 1</td>
<td>14.00 mg</td>
</tr>
<tr>
<td>Salicylic acid 1.0 g L&lt;sup&gt;-1&lt;/sup&gt;−7.24 mM</td>
<td>SA 2</td>
<td>140.00 mg</td>
</tr>
<tr>
<td>Chitosan oligosaccharide&lt;sup&gt;a&lt;/sup&gt; 0.1 g L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>COS 1</td>
<td>14.00 mg</td>
</tr>
<tr>
<td>Chitosan oligosaccharide&lt;sup&gt;a&lt;/sup&gt; 1.0 g L&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>COS 2</td>
<td>140.00 mg</td>
</tr>
<tr>
<td>Sandblasting</td>
<td>SB</td>
<td>× 5</td>
</tr>
</tbody>
</table>

The cumulative treatment was calculated as the amount of compound applied per plant during one or over several treatments.

<sup>a</sup>The molecular weight of mixed product was unknown.

(S7401; Sigma Aldrich) at 0.1 and 1 g L<sup>-1</sup> aqueous solutions, HP (Matos, 10%) at 0.1 and 1.0%, COS (provided by Dalian Glycobio Ltd) in the aqueous solutions 0.1 and 1.0 g L<sup>-1</sup>, and SB (Bödeger, Model 260-3, aluminium oxide particles). A manual garden vaporizer (Gardena) delivered 1 mL of liquid per spray, and 24 sprays covered the majority of a plant in a water film. To compensate for the growth during the 5-week treatment period, two additional sprays were added each week (32 sprays at last treatment). Sandblasting was carried out for 2 × 15 s at the onset of the experiment and for 2 × 20 s at the last treatment (see Table 1 for an overview of treatments). Treatments were carried out during the late afternoon to minimize any sun scalding effect from water droplets on the leaf surface. No physical changes were observed after the treatments, apart from the highest dose of SC, which caused reddened midribs in leaves of some plants. Lower leaves and upper leaves were marked and defined as in Ex1 (Fig. 1). At sampling, the lower leaves and the upper leaves were collected and immediately placed into plastic bags between filter papers, soaked with ice-cold water and stored at 3°C.

**Fluorescence microscopy and picture analyses Ex1**

Leaf samples were analysed under an Olympus BH2 microscope equipped with a 100-W mercury fluorescence lamp at ×40 magnification using a standard broad BG filter and a Canon EOS 10D camera. From each sample, the adaxial side of three leaf tips was exposed and two predefined areas from each of these were photographed, giving a total of six photographs per sample. Photographs were analysed using NIS-elements BR 3.1 software (Nikon Instruments). On each photograph, an area of 0.2–0.9 mm<sup>2</sup> was measured exactly and the number of GT within the area was counted. Four GT from each area were randomly selected and the lengths were measured. One entire leaf from each sample was photographed and the area was quantified using WinRHIZO 5.1 (Regent Instruments).
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Fluorescence microscopy and picture analyses Ex2
Leaf samples were analysed under a Nikon AZ100 microscope equipped with a 200-W fluorescent light source and a short-pass fluorescein isothiocyanate filter (Ex 465–495/DM 505/Ba 515–555) at ×40 magnification. The abaxial side was exposed, and within the first major secondary lobe from the leaf tip, three areas were photographed using the ‘binning’ option, exposure times of 2 s and Gain 1 (Fig. 2). Pictures were analysed with NIS-elements BR 3.1 (Nikon Instruments). Bright light intensity was used to discriminate the GT from the background, and false positives were excluded by size and shape recognition. From each of the three pictures derived from the same leaf, two areas (0.3–2.4 mm²) were marked, and GT counts and size parameters based on pixel counts were recorded.

Data analyses
Statistical analyses were performed in R ver. 2.11.1 (R Development Core Team 2010). Graphs were prepared in Sigmaplot (2000 for Windows, ver. 6.00). Stress effects were analysed in a linear mixed model approach (lmer, lme4 package). Statistics for Figs 3–5 were performed in linear mixed models by relating the desired variables (GT density, GT area, GT length, GT width, leaf area and height of plant) to the fixed variable ‘Treatment’ and adding the relevant random effects. Probabilities of significance were given as $P > |t|$ in an MCMC algorithm. Statistical analyses of data from the adaxial and abaxial leaf sides (Table 2) were performed as paired t-tests, and differences between sampling times (Fig. 6) were performed as honestly significant difference (HSD) Tukey’s tests.

Results
In Ex1, the densities of GT on upper leaves treated with SA (all concentrations) and COS were significantly lower than those in control leaves (Fig. 3A). Lengths of GT on treated upper leaves were not significantly different from the control, but it is noteworthy that almost
Fig. 3 Effects of stress treatments in the field experiment (Ex1) on trichome density (A), trichome length (B), leaf area (C) and calculated trichome density of entire leaves (D). Black columns are results from upper leaves (not initiated at onset of treatments) and grey columns are results from lower leaves (fully developed at onset of treatments). Error bars represent the SEM ($n = 9$) and an asterisk indicates significant difference from control ($P < 0.05$) in a linear mixed model. Abbreviations of treatments are given in Table 1.

Fig. 4 Effects of stress treatments in greenhouse experiment (Ex2) on trichome density (A), trichome length (B), trichome width (C) and trichome area (D). Black columns are results from upper leaves (not initiated at onset of treatments) and grey columns are results from lower leaves (fully developed at onset of treatments). Error bars represent the SEM ($n = 18$) and an asterisk indicates significant difference from control ($P < 0.05$) in a linear mixed model. Abbreviations of treatments are given in Table 1.
all mean values of stress-treated leaves were lower than the control (Fig. 3B). In the lower leaves, no significant effects of the treatments on either the densities or the length of GT were observed, though most treated plants had a lower mean length of GT as compared with controls (Fig. 3A and B). Treatment with SA1, SA2, JA2, COS, SB×4 and Cut×4 produced significantly larger leaf areas than the control (Fig. 3C). Multiplying the values of GT densities with the leaf areas produced a data set of calculated GT per leaf (Fig. 3D). None of the calculated GT per leaf values showed a significant relationship to the treatments. Neither the final height nor the increment in height during the experiment was significantly affected by the treatments (data not shown).

In Ex2, GT densities on upper leaves of treated and control plants were not significantly different, though it is noteworthy that the mean densities of the control plants were lower than those of most of the treated groups (Fig. 4A). Two treatments (SC1 and SB) showed significantly smaller areas and widths of GT in upper leaves compared with the control (Fig. 4C and D). Densities of GT in the lower leaves showed significantly lower densities in treated leaves in SA1 and SB, and mean values of all the remaining treated groups were non-significantly lower than controls (Fig. 4A). Compared with control plants, the area, length and width of GT of the lower leaves were significantly lower in several of the treated plants, and non-significantly lower in the remaining groups (Fig. 4B–D). Neither the final plant height nor the increment in height during the experiment was significantly affected by the treatments (data not shown). Plants of two of the three clones in Ex2 (Clones 1 and 3) were infected by a powdery mildew ( Golovinomyces sp.). The growth of the infected plants between propagation and the onset of treatments was affected, and infected plants were significantly ($P < 0.0001$) shorter than uninfected plants within the same clone (data not shown). At the end of the experiment, this difference in height was non-significant. The level of fungus infection was scored (levels: 0, 1 or 2) and the random effect caused by the infection was included in the linear mixed models.

Analysing values from the fungus-free Clone 2 clone (Fig. 5) eliminated the variation caused by the fungal infection. Glandular trichomes densities in the upper leaves were significantly higher after treatment with SC2, HP2 and COS2 as compared with control plants, and the remaining treatments produced non-significantly higher mean values than control plants. Treatments tended to result in non-significantly lower mean values of GT densities in lower leaves compared with control plants (Fig. 5A). Area, width and length of the GT on the upper leaves were not significantly
Table 2: Trichome densities and trichome length on adaxial and abaxial leaf sides of control plants of the field experiment (Ex1) and greenhouse experiment (Ex2).

<table>
<thead>
<tr>
<th></th>
<th>Adaxial</th>
<th>Abaxial</th>
<th>Adaxial</th>
<th>Abaxial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex1 Upper leaves</td>
<td>32.4 ± 1.8**</td>
<td>39.1 ± 2.5</td>
<td>66.1 ± 0.9 NS</td>
<td>65.2 ± 0.8</td>
</tr>
<tr>
<td>Ex1 Lower leaves</td>
<td>23.7 ± 3.0**</td>
<td>31.7 ± 3.1</td>
<td>65.2 ± 0.7 NS</td>
<td>66.3 ± 0.8</td>
</tr>
<tr>
<td>Ex2 Upper leaves</td>
<td>8.1 ± 0.9**</td>
<td>13.0 ± 1.7</td>
<td>64.0 ± 1.5 NS</td>
<td>62.2 ± 1.1</td>
</tr>
<tr>
<td>Ex2 Lower leaves</td>
<td>5.9 ± 1.2*</td>
<td>9.3 ± 1.1</td>
<td>60.2 ± 2.2 NS</td>
<td>59.3 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means of mean values from six photographs per sample ± SEM (n = 5 (Ex1) and n = 9 (Ex2)). Asterisks indicate significant difference between adaxial and abaxial values (NS, not significant; *P < 0.05, and **P < 0.01) in paired t-tests.

Fig. 6: Trichome density (A) and trichome length (B) of untreated plants from the field experiment (Ex1) sampled at weekly intervals over 5 weeks. Black columns show upper leaves (sampled at progressively higher positions of the main stem) and grey columns show the lower leaves (sampled at the same position throughout the experiment). Lower-case letters show significant differences (P < 0.05) in a HSD Tukey’s test. Error bars represent the SEM (n = 9).

Observation of untreated control plants from Ex1 over 5 weeks (Fig. 6A) revealed constant GT densities in the lower leaves (sampled at the same position on the plant), whereas the upper leaves (sampled at progressively later initiation positions) had a non-significant tendency to produce higher GT densities. The lengths of GT (Fig. 6B) of both lower leaves and upper leaves were significantly larger at Week 2 compared with other weeks. These measurements coincided with the precipitation pattern of the period, as heavy rains saturated the soil before Week 2, and Weeks 4 and 5 had light rainfalls. Prior to Weeks 1 and 3, conditions were dry and windy. Investigation of the adaxial and abaxial sides of leaves of control plants (Table 2) revealed that densities of GT on the abaxial side were significantly higher than those on the adaxial side. Length of GT did not differ significantly between the two sides.

Discussion

Hypotheses

It was hypothesized that the applied stress on full-grown A. annua plants would result in higher GT densities and larger GT in developing leaves (upper leaves), and that GT on mature leaves (lower leaves) would remain unchanged. The stress treatments induced higher densities of GT only in some treatments on newly developed leaves (upper leaves) of Clone 2 from Ex2 (Fig. 5A). However, the stress treatments did not increase the size of any GT relative to the control. On the contrary, several treatments in Ex2 resulted in significantly smaller GT in the lower leaves in response to stress, and similar non-significant trends were observed in the remaining lower leaves of both Ex1 and Ex2. Thus, in this study, the hypothesis that stress is able to initiate higher GT densities in upper leaves of large plants could only be confirmed in a limited subset of the

affected by the treatments, but on the lower leaves there were several significantly lower size values as compared with controls (Fig. 5B–D).
plants, and the hypothesis that stress will induce larger GT was rejected. Reports in the literature concluding that GT morphology in young A. annua plants is relatively plastic in response to stress treatments were not confirmed in mature plants grown under exposed or protected conditions.

**Effect of stress on GT density**

The two stress-based experiments showed very limited effects on GT densities of newly developed leaves (upper leaves) of adult plants. Only the upper leaves of Clone 2 in Ex2 showed a significant increase in GT densities following repeated application of the strongest doses of SC, HP and COS. This difference in response may be partly related to differences in the impact of uncontrolled stress between and within the two experiments. The relatively heterogeneous appearance of plants of Ex1 was probably related to the harsh and variable outdoor environmental conditions, whereas the plants of Ex2 were protected in a greenhouse. Clones 1 and 3 of Ex2 included many individuals infected by powdery mildew, which significantly affected their height and growth pattern. The mildew-free individuals of Clone 2 were the least affected by factors other than the stress treatments, and these plants were visibly more uniform in growth. The results indicated that stress treatments could induce small changes in GT densities, but that the effect could easily be overwhelmed by other factors. These factors included stress by environmental conditions, interplant variation in density of GT, physiological age, proximity to flowering, genetic predispositions and how 'saturated' the leaf was with GT at the time of stress treatment. This 'saturation' effect was reported by Moes et al. (2011), who found a lower ability to increase GT densities in plants which initially had higher GT densities. There is limited information available on the possible change in this plasticity in response to stress when plants grow larger or approach the onset of flowering. Holeski (2007) demonstrated that the GT density on the fifth leaf of M. guttatus was more responsive to damage than the seventh leaf. The initial density of GT is shown to be a heritable trait (Graham et al. 2010), and possibly, though not documented in the literature, the plasticity in the GT density response to stress is similarly heritable.

The differences in results of GT densities in Ex1 and Ex2 illustrated the variability and complexity in GT morphology of adult A. annua. Interestingly, the GT densities of upper leaves in Ex1 in several stress treatments (Fig. 3A) were decreased, as opposed to the increased densities of upper leaves observed in several treated groups of Ex2. This might be explained by the significant effect that several treatments had on leaf size of upper leaves of Ex1 (Fig. 3C). When leaf size increases, there is an implied 'dilution' of GT due to the increased expansion of treated leaves as compared with controls. When applying a method from Lommen et al. (2006) to calculate the total GT number per leaf (Fig. 3D), we determined a non-significant effect of stress on the density of GT in Ex1.

**Salicylic acid** This was applied in both Ex1 and Ex2. The significant decrease in GT density on upper leaves of Ex1 (Fig. 3A) was possibly due to the dilution effect mentioned above. The upper leaves of the overall Ex2 (Fig. 4A) showed no significant response in GT density to SA, and Clone 2 of Ex2 showed a limited, but non-significant, increase in GT density in the SA2 treatment. The effect of SA on GT densities of A. annua is undocumented, but Traw and Bergelson (2003) showed no effect of SA on the density of GT of A. thaliana.

**Jasmonic acid** This was applied only in Ex1, and no significant differences were found in GT densities on the upper leaves as compared with the controls. Moes et al. (2011) reported a several-fold increase in GT density in young A. annua plants after applying JA and Liu et al. (2009) reported similar significant increases in GT densities following treatment with JA. In the present study treatments were applied to older plants, which may already have been saturated by relatively high GT densities or had lost phenotypic plasticity in the ability to respond to stress.

**Chitosan oligosaccharide** Plants were treated with COS in both Ex1 and Ex2. The significant decreases in GT density in upper leaves of Ex1 (Fig. 3A) were possibly due to the dilution effect of leaf expansion mentioned above. The upper leaves of the overall Ex2 (Fig. 5A) showed no response to COS, but the upper leaves of Clone 2 in Ex2 (Fig. 6A) showed a significant increase in the density of GT in response to the COS2 treatment, and a non-significant increase in the COS1 treatment. The effect of COS on GT densities in A. annua is undocumented in the literature, but Lei et al. (2011) reported a significant increase in both AN and HP levels in leaves after foliar application of COS.

**H₂O₂** The upper leaves of the overall Ex2 (Fig. 5A) showed no response to HP, but the isolated Clone 2 (Fig. 6A) showed a significant increase in the HP2 treatment, and a non-significant increase in the HP1 treatment. The effect of HP on GT densities in A. annua is undocumented in the literature.

**NaCl** The upper leaves in Ex2 overall (Fig. 5A) showed no response to SC, but the upper leaves of Clone 2 in Ex2 (Fig. 6A) showed a significant increase in the
density of GT in response to the SC2 treatment, and a non-significant increase in the SC1 treatment. The effect of foliar application of SC on GT densities in *A. annua* is previously undocumented in the literature.

**Artificial wounding** Wounding from SB in both Ex1 and Ex2 and from scissor cutting (Cut) in Ex1 failed to raise GT densities. The effect of wounding on GT densities in *A. annua* is previously undocumented in the literature, but Traw and Bergelson (2003) and Travers-Martín and Muller (2008) showed a significant increase in GT density in response to leaf wounding in *A. thaliana* and *Sinapis alba* (both Brassicaceae), respectively.

**Effect of stress on the size of GT**

Moës et al. (2011) observed a significant increase in the area of GT following treatment with JA and gibberellic acid, and a significant decrease in the GT area in cytokinin-treated leaves of young individuals of a low-AN-producing *A. annua* cultivar. They interpreted the increase in GT area to be linked with a more advanced maturation stage of the GT and/or increased biosynthetic activity, and that the decreased areas were linked with cytokinin maintaining cells in division rather than proceeding towards maturity. In the present study, decreased sizes (area, length or width) of GT in stress-treated plants were observed for both upper leaves and lower leaves in Ex1 (Fig. 3B), and the decreased GT sizes in the lower leaves were significant for most treated plants in Ex2 (Fig. 4B–D) and Clone 2 (Fig. 5B–D). In contrast to Moës et al. (2011), we interpreted the smaller GT as an indication that the GT of stressed plants were more mature than the GT of the control plants. How stress affects the maturation of GT thus remains unresolved, though it is clear that stress had an effect on the size of GT. In our observations, no collapsed GT or any remnants of GT were observed. The present study leads to the theory that not only proximity to flowering, but also the influence of stress, may promote the maturation of GT. Clearly, more knowledge is needed on the maturation and rupture processes of GT in *A. annua*.

Both Pu et al. (2009) and Aftab et al. (2010) applied SA as a 1 mM spray on young *A. annua* plants, JA was applied by Liu et al. (2009) as a 1 mM spray and by Moës et al. (2011) as a combination of a 0.1 mM foliar spray and substrate application. Lei et al. (2011) applied COS as a 100 mg L⁻¹ foliar spray. All these stress treatments caused significant changes in GT morphology or SM content. For SC, HP and wounding, no comparable studies of *A. annua* were found in the literature. In the present study, the chosen doses and frequencies of treatments (Table 1) reflected what had previously been shown to be effective in the literature but doses were increased in proportion to the physically larger plants. The frequency and degree of application of stress differed between Ex1 and Ex2, as only the upper part of the plants of Ex1 were treated once, whereas the entire plants were treated five times in Ex2.

**General observations**

Mean GT densities within treatment groups were 20–37 GT mm⁻² for the field experiment (Ex1) (adaxial side) and 10–15 GT mm⁻² in the greenhouse experiment (Ex2) (abaxial side). These ranges corresponded well to the 5–37 GT mm⁻² reported by Arsenault et al. (2010), the 12–75 GT mm⁻² by Moës et al. (2011), the 20–75 GT mm⁻² by Lommen et al. (2006) and the 2–80 GT mm⁻² by Graham et al. (2010). Other studies have reported different GT densities. For example, Kapoor et al. (2007) reported 80–140 GT cm⁻² (corresponding to only 0.8–1.4 GT mm⁻²) and Hu et al. (1993) reported 190–225 GT mm⁻². Mean values of the GT length and width for the present study were in the ranges of 60–65 and 30–33 μm, respectively. In the literature, only Hu et al. (1993) presented GT dimensions of *A. annua* and reported results of 45–48 and 27–30 μm, respectively. Mean values of projected GT areas were in the range of 1700–2300 μm², as measured by bright pixels in fluorescence microscope images. By estimating GT area from graphs presented by Moës et al. (2011), a range of 400–1100 μm² was determined. Differences between these ranges could be attributed to the fact that Moës et al. (2011) studied very young plants and areas were calculated by using the formula Lwxw/4. When we applied the same formula to our data, estimated values showed a 20–30 % lower GT area than the projected values.

None of the leaves that were stress treated at an already developed stage (lower leaves) showed increases in GT density as compared with controls with similar leaf sizes (Figs 3A, 4A and 5A). The present study thus supported the general understanding that GT of *Artemisia* are only initiated in leaves at an early developmental stage (Ascensão and Pois 1987—*A. campestris*; Duke and Paul 1993—*A. annua*). Investigation of control plants during a 5-week period (Fig. 6A) showed constant densities of GT on lower leaves, whereas GT densities of upper leaves increased slightly, but non-significantly. Similar trends were observed in *A. annua* by Lommen et al. (2006) and Liu et al. (2009), and in *M. arvensis* by Shanker et al. (1999). The significant differences in length of GT observed in both upper leaves and lower leaves during the 5-week study (Fig. 6B) followed the precipitation pattern closely, and it was proposed that the length of GT was a flexible parameter linked to the water status of the leaf.

The comparison of GT on the adaxial and abaxial sides of the leaf in untreated plants of Ex1 and Ex2 (Table 2)
showed significantly higher densities of GT on the abaxial than the adaxial side. Ascensão and Pais (1987) found a similar trend on leaves of \textit{A. campestris}, whereas Hu et al. (1993) found the opposite trend in \textit{A. annua}. Kelsey and Shafizadeh (1980) reported no difference between the adaxial and abaxial GT density in \textit{A. nova} and, based on estimations of graphs of GT densities on vegetative leaves of \textit{A. annua}, neither did Arsenault et al. (2010). In the present study, the length of the GT did not differ significantly from the abaxial to the adaxial side of the leaf irrespective of leaf age.

Several researchers have shown that various types of stress can increase the content of AN and other SM in \textit{A. annua} (Liersch et al. 1986—chloroform; Qureshi et al. 2005—oxidative stress; Ferreira 2007—potassium deficiency; Qian et al. 2007—salinity; Kapoor et al. 2007—mcyarrhiza; Özgüven et al. 2008—nitrogen; Pu et al. 2009—SA; Liu et al. 2009—JA; Mannan et al. 2010—dimethyl sulphide; Liu et al. 2010—wounding; Lei et al. 2011—chitosan; Maes et al. 2011—JA; and Nguyen et al. 2011—review). Although differences in the developmental age and size of the investigated plants have to be taken into account, the present study suggested that stress may increase the AN production or conversion of AN precursors within existing GT, rather than directly influencing the number or size of GT.

**Conclusions and forward look**

The results showed very little impact of applied stress on the GT densities of leaves initiated after or under the influence of stress (i.e. the upper leaves). Only a subset of plants in a greenhouse experiment (Ex2) produced significantly higher GT densities on upper leaves in response to multiple treatments of SC, HP or COS at high concentrations. The density of GT on older (i.e. lower) leaves that had already developed at the time of treatment was largely unaffected by stress. The limited effect on upper leaves, especially in Ex1, compared with previous reports is attributed to the larger size of the plants, interference by uncontrolled environmental stresses in the field, and a possible ‘saturation’ effect of GT in this study. The size of GT was largely unaffected on the upper leaves of Ex2, but several stress treatments decreased GT size on the lower leaves, probably as an outcome of accelerated maturation. The results of this study showed that it was not possible to induce similar stress responses on GT in full-grown plants grown under less protected conditions as found in previous research on young plants. Future biochemical analyses of plant samples from the present experiments are needed to examine this relationship further.

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**Contributions by the authors**

A.K. executed the experiments and analyses, and wrote the paper in collaboration with K.G. and M.J.

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**Conflict of interest statement**

None declared.

**References**


8. PAPER II

SUBMITTED

“Artemisinin production and precursor ratio in full grown *Artemisia annua* L. plants subjected to external stress”

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Artemisinin production and precursor ratio in full grown *Artemisia annua* L. plants subjected to external stress

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**Abstract**

The concentration of the lifesaving antimalarial compound artemisinin in cultivated *Artemisia annua* plants is relatively low, and thus research in improving the content is important. In the present study external stress was applied to adult plants of *Artemisia annua* and the effect was examined on the concentrations of artemisinin and its immediate precursors in leaves, and concentrations were related to the densities and sizes of glandular trichomes. Plants were stress treated weekly for five weeks by sandblasting or spraying with salicylic acid, chitosan oligosaccharide, \(H_2O_2\), and NaCl solutions. Contents of AN related compounds were analysed in leaf samples from an upper and a lower position of the plants, and glandular trichomes were quantified and measured. In lower leaves, several stress treatments had significantly negative effects on concentrations of AN related compounds, whereas the ratios between compounds showed an increased conversion to artemisinin. In the upper leaves no changes were observed compared to controls. Linear relations were found between the content of metabolites and the density of trichomes in both upper and lower leaves, and size of trichomes in lower leaves. Results suggested that older and younger leaves may respond differently to applied stress. Some plants were infected by powdery mildew, and this caused significantly different compositions of the AN related compounds, compared to uninfected plants. In conclusion, stress induced changes in concentrations of artemisinin related compounds seemed largely to be explained by changes in trichome densities and sizes.

**Keywords:** Artemisinin, *Artemisia annua*, Leaf age, Stress, Trichome area and density.

**Abbreviations:**

AN, artemisinin; DHAA, dihydroartemisinic acid; AA, artemisinic acid; DHAAA, dihydroartemisinic aldehyde; AAA, artemisinic aldehyde; DHAAOH, dihydroartemisinic alcohol; AAOH, artemisinic alcohol; AN-c, artemisinin related compounds, including AN; GT, glandular trichomes; *A. annua*, *Artemisia annua*; SA, salicylic acid; COS, chitosan oligosaccharide; SB, sandblasting.
Chapter 8: Paper II

Introduction

Glandular trichomes (GT) on Artemisia annua (A. annua) leaves are the site of production and storage of the anti-malarial compound artemisinin (AN) and other secondary metabolites with several pharmaceutical and industrial uses. The economically important sesquiterpene lactone AN has only been found naturally occurring in A. annua (Brown, 2010; Effert et al., 2011). Attempts to synthesize AN or produce AN in engineered yeast and other bioengineered target organisms have proven difficult and until now economically not viable (Ro et al., 2006; Covello, 2008; Graham et al., 2010). So in recent years, a considerable interest has focussed on understanding the natural biosynthetic pathways of AN, the enzymes involved, and the underlying genetic expressions. The majority of the enzymatic and non-enzymatic steps in the pathway are now documented (Bouwmeester et al., 1999; Wallaart et al., 1999; Bertea et al., 2005; Nguyen et al., 2011), and the understanding of the genetic background of the pathway is under elucidation (Arsenault et al., 2010; Graham et al., 2010; Olofsson et al., 2011). The present study was based on the proposed biosynthetic pathway (Fig. 1), in which AA does not acts as an indirect precursor to AN. Wallaart et al. (2001) presented two different chemotypes of A. annua; Type I, containing relatively high levels of AN and dihydroartemisinic acid (DHAA) and low levels of AA, and Type II, containing relatively high levels of artemisinic acid (AA) and low levels of AN and DHAA. Maes et al. (2011), and Wu et al. (2011) demonstrated that a range of phytohormones initiated two different chemotypic expression patterns of key genes involved in the biosynthetic pathway of AN.

The biseriate capitate GT of A. annua are composed of ten cells stacked in pairs (Duke and Paul, 1993; Nguyen et al., 2011). The four lower cells primarily function as stalk for the six upper cells, which all border a secretory cavity and contribute to the filling of this apical subcuticular space with secondary metabolites. AN is phytotoxic to the A. annua plant itself and other plants (Duke et al., 1987), and it has been proposed that to protect the plant from the toxic effect, the biosynthesis of AN only takes place in the GT (Duke et al., 1994). This makes the GT a key focus area when working with stress effects on AN related compounds (AN-c, including AN). A recent laser dissection study of GT from A. annua showed that within the GT, the key enzymes of AN production were expressed in both the apical and the subapical cells of the GT (Olofsson et al., 2012), and not just in the two apical cells, as previously reported (Olsson et al., 2009).

For the A. annua plants, the secondary metabolites are part of the defence against herbivory, pathogens and other disadvantageous conditions (Hu et al., 1993; Duke and Paul, 1993; Duke, 1994). When defining “stress” as growth conditions that differ from “normal” conditions for the plants, research has demonstrated that applied stress can affect the biosynthetic pathways of AN, and the concentrations of AN and other SM in A. annua (Liersch et al. 1986; Woerdenbag et al., 1993; Qureshi et al., 2005; Ferreira, 2007; Qian et al., 2007; Chaudhary et al. 2008; Öżgüven et al., 2008; Pu et al. 2009; Liu et al., 2009; Mannan et al., 2010; Liu et al., 2010; Lei et al., 2011; Aftab et al., 2010; Maes et al., 2011; Rai et al., 2011; Aftab et al., 2011; Banyai et al., 2011). Though the experiments were quite different in design and execution, a common feature was, that a very diverse range of treatments resulted in changes in the concentrations of AN. This indicates that the A. annua plants possess the ability to respond to applied disturbances from their “normal” balance. Changes in the production of AN and other secondary metabolites in A. annua
Fig. 1. Biosynthetic pathways of AN. Full arrows indicate conversions commonly accepted in the literature, and dotted lines indicate conversions, which remain controversial. Pie charts illustrate the average proportional concentration in control plants, upper pie chart = upper leaves, lower pie chart = lower leaves.
in response to external stress may be attributed to phenotypic plasticity resulting in an increased density of GT as demonstrated in several investigations (Liersch et al., 1986; Kapoor et al., 2007; Liu et al., 2009; Maes et al., 2011; Kjaer et al., 2012). Maes et al. (2011) further determined that a cultivar with a low AN content had a greater ability to undergo phenotypic plasticity by changing the GT density than a cultivar with higher AN content, suggesting the existence of a mechanism of a maximum number of GT on the leaves. It has been suggested that younger plants are more likely to undergo phenotypically changes in GT in response to external stress than older plants (Kjaer et al., 2012). Nguyen et al. (2011) presented an overview of the overall knowledge on the influence of external factors on the production of GT and AN in A. annua.

It has been demonstrated that leaves that develop later in the lifetime of the plant have higher densities of GT than those initiated earlier (Ferreira and Janick, 1996; Arsenault et al., 2010; Kjaer et al., 2012). A gradual conversion of AN precursors to AN occurs in the GT during the maturation of GT (Lommen et al., 2006; Nguyen et al., 2011), and proximity to flowering further increased the AN content (Ferreira et al., 1995; Lommen et al., 2006; Ma et al., 2008; Baraldi et al., 2008; Arsenault et al., 2010). When leaves turned brown and died on the plant, a radical change in the proportions between AN and its precursors in favour of AN was observed (Lommen et al., 2007). It was hypothesised that oxidative stress and possibly the collapse of trichomes during leaf aging induced a higher conversion of DHAA to AN. A similar trend was reported when different drying strategies were applied to harvested plants (Ferreira and Luthria, 2010). This makes it relevant to observe the effect of stress on leaves at different developmental stages.

The aim of this study was to apply stress to full grown plants in a greenhouse, and describe the effect on the concentration and composition of AN-c, and to relate these responses to phenotypical changes in the density and size of GT (data from Kjaer et al., 2012). An unintended infection with a powdery mildew (Golovinimyces sp.) provided the possibility to preliminarily elucidate the influence of a fungus infection on the concentrations of, and ratios between AN-c. The following hypotheses were tested for both the upper leaves and lower leaves of uninfected plants: (1) Stress will cause an elevated individual and total concentration of AN-c, (2) Stress will change the ratio between concentrations of AN-c, (3) Density of GT will be positively related to individual and total concentrations of AN-c, (4) Density of GT will be related to ratios between concentrations of AN-c, (5) Size parameters of GT will be positively related to individual and total concentrations of AN-c, (6) Size parameters of GT will be related to ratios between concentrations of AN-c.

Materials and methods

Plant material

From a field population of seed propagated A. annua (cv. Artemis, F2 seeds, Mediplant, Switzerland), three plants were randomly selected as mother plants. The plants were estimated to be chemotypically of Type I, because the samples contained high levels of AN and DHAA as compared to levels of AA (see results). A population of clonally propagated plants were obtained by cuttings from the mother plants during two rounds of multiplication in a greenhouse. Tip cuttings were ca. 10 cm long and consisted of 4-5 internodes longer than 1 cm. Cuttings were rooted after 2-3 weeks, and potted in 3.5 L containers with Pindstrup No 2 peat moss (Pindstrup Mosebrug, Denmark). Plants were
transferred from the nursery to the experimental greenhouse, and allowed to acclimatize for 7 days before the onset of treatments. Plants were 9 weeks from propagation and 80-110 cm tall at the onset of the treatments and 150-190 cm at sampling of leaves. During the experiment, plants were drip irrigated twice diurnally with a liquid fertilizer adapted for Asteraceae cultivation. Night temperatures ranged from 8 to 12°C and day temperatures from 10 to 32°C.

**Mildew infection**

In the field from which the three mother plants were collected, a few plants were observed to be completely covered by mildew (Fig. 2). The collected mother plants appeared to be uninfected, but when plants were propagated and re-propagated in a greenhouse, patches of mildew infection started to develop and spread among neighbouring plants. Due to the risk of introducing an uncontrolled additional stress treatment to the plants, the use of fungicides was avoided. Instead the degree of infection was visually recorded both at the time of treatment and sampling, and scored as: uninfected, medium infected, or severely infected. At the beginning of treatments all plants of clones 1 and 2 were scored as uninfected, and most plants of clone 3 were medium or severely infected. At leaf sampling five weeks later, most plants no longer had visual infections, and only few individuals of clone 3 were still scored as medium infected. The fungus was identified to the genus *Golovinomyces*, the species was not determined (Mørk, 2011).

![Fig. 2. Plants showing no (left) and severe (right) infection by *Golovinomyces* sp.](image)

**Experimental design**

The experiment was carried out during April-May, 2010. A randomized complete block design with subsampling was set up in a greenhouse divided into two self-contained compartments. In each compartment, three beds of plants were established, each bed consisting of 3 × 23 plants of one of the three clones. Plants were 50 cm apart and individual beds were separated by 100 cm of gravel. Every second row of three plants were left as guard rows, allowing for 11 treatable subplots of three plants in each bed (2 blocks × 3 clones × 3 plants = 18 subplots per treatment). Stress treatments were performed once a week for five weeks, and included spraying with NaCl (Salina) in 10 g L⁻¹ aqueous solution, salicylic acid (SA) (S7401, Sigma Aldrich) in 1 g L⁻¹ aqueous solution, H₂O₂ (Matas,
10% 1.0%, chitosan oligosaccharide (COS) (provided by Dalian Glycobio Ltd. China) in 1.0 g L\(^{-1}\) aqueous solution, and sandblasting (SB) (Badger, Model 260-3, aluminium oxide particles). A manual garden vaporizer (Gardena) delivered 1 mL liquid per spray, and 24 sprays covered the majority of a plant in a water film. To compensate for the growth during the five week treatment period, two additional sprays were added each week (32 sprays at last treatment). Sandblasting was carried out for 2 × 15 seconds at the onset of the experiment and 2 × 20 seconds at the last treatment. Treatments were carried out during the late afternoon to minimize any sun scalding effect from water droplets on the leaf.

Fig. 3. Schematic representation of treatment and sampling. The upper leaf developed during the influence of treatments. The lower leaf was positioned 10 internodes down the main axis below the upper leaf at 0 days. Lower leaves were assumed to be fully expanded at the beginning of the treatments. Leaves marked as white in figure were collected and pooled for analysis of artemisinin related compounds.
surface. No physical changes were observed after the treatments, apart from NaCl, which caused reddened midribs in leaves of some plants. The upper leaf was defined as the first leaf below the apex on the main stem with internodes longer than 2 cm (Fig. 3). To recognize leaves, which were at a comparable developmental stage at the onset of the experiment, the main stem below the tenth internode from the upper leaf was marked on all the plants at the day of the first treatments. The leaf above this mark was the lower leaf. Seven days after the last treatment, the lower leaves and the upper leaves were collected for GT analyses and immediately placed into plastic bags between filter papers, soaked with ice cold water and stored at 3°C. In addition, samples of four leaves were harvested near each of the upper leaves and lower leaves (two above and two below) for analysis of AN metabolites (Fig. 3). Samples were frozen at -20°C, freeze dried for 48h, and stored at -20°C until extractions.

Analyses of GT

Leaf samples were analysed under a Nikon AZ100 microscope equipped with a 200W Fluorescent light source and a short pass FITC filter (Ex 465-495/DM 505/Ba 515-555) at ×40 magnification. The abaxial side of leaves was exposed, and within the first major secondary lobe from the leaf tip, 3 areas were photographed using the ‘binning’ option, exposure times of 2 seconds and Gain 1. Pictures were analysed with NIS-elements BR 3.1 (NIKON instruments). Bright light intensity was used to discriminate the GT from the background, and false positives were excluded by size and shape recognition. From each of the 3 pictures deriving from the same leaf, 2 areas (0.3-2.4 mm²) were marked, and GT counts and size parameters based on pixel counts were recorded. Data analyses were described in Kjaer et al. (2012) and part of the resulting data were included in this study.

Extraction of artemisinin and related compounds for analyses

Dry plant material was homogenized in a mixer mill (MM200; stainless steel ball Ø=7 mm; Retsch, Haan, Germany) and about 50 mg accurately weighed into an Eppendorf vial with 1.0 ml MeOH (HPLC grade; Th. Geyer, Germany), which was then briefly vortexed. The tubes were sonicated for 5 min. and centrifuged at 14000 rpm for 5 min. The supernatant was collected and filtered through a 0.45 μm syringe filter into a brown HPLC glass vial. The pellet was re-dissolved in 0.5 mL MeOH and the procedure of sonication and centrifuging was repeated. The supernatant was again filtered into the vial, and stored at -20°C. Before measurement the samples were diluted 100 times with 20% acetonitrile in water (Biosolve, Valkenswaard, The Netherlands) containing 0.1% formic acid (Sigma-Aldrich, The Netherlands).

Detection and quantification by UPLC-MRM-MS

Targeted analysis of artemisinin and pathway intermediates was performed with a Waters Xevo tandem quadrupole mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was obtained on an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters) by applying a water/acetonitrile gradient to the column, starting from 5% (v/v) acetonitrile in water for 1.25 min and rising to 50% (v/v) acetonitrile in water in 2.35 min, followed by an increase to 90% (v/v) acetonitrile in water in 3.65 min, which was maintained for 0.75 min before returning to 5% acetonitrile in water using a 0.15 min gradient. Finally, the column
was equilibrated prior to the next injection for 1.85 min using 5% acetonitrile in water. Operation temperature and flow rate of the column were 50°C and 0.5 mL min⁻¹, respectively. Injection volume was 5 μL. The mass spectrometer was operated in positive electrospray ionization mode. Cone and desolvation gas flows were set to 50 and 1000 L h⁻¹, respectively. The capillary voltage was set at 3.0 kV, source temperature at 150°C, and desolvation temperature at 650°C. The cone voltage was optimized for the different compounds using the Waters IntelliStart MS Console. Argon was used for fragmentation by collision-induced dissociation in the ScanWave collision cell. Quantification of compounds was done by multiple reaction monitoring (MRM). The optimized settings for MRM measurements are listed in Table 1. Both arte misinin and DHAA were a gift from Dafra Pharma (Belgium). DHAA was used by Chiralix (Nijmegen, the Netherlands) to synthesize the other precursors that were checked by NMR and were more than 98% pure. The reference standards were used for external calibration curves.

Table 1. The optimized settings for UPLC-MRM-MS measurements.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Parent (m/z)</th>
<th>Daughter (m/z)</th>
<th>Cone voltage</th>
<th>Collision voltage</th>
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<tbody>
<tr>
<td>AAA</td>
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<td>145.08</td>
<td>18</td>
<td>16</td>
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<tr>
<td></td>
<td></td>
<td>159.09</td>
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<td></td>
<td></td>
<td>210.2</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>DHAAA</td>
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<td>105.14</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>203.2</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>AAOH</td>
<td>221.16</td>
<td>147.09</td>
<td>14</td>
<td>20</td>
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<tr>
<td></td>
<td></td>
<td>203.27</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>DHAAOH</td>
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<td>95.07</td>
<td>14</td>
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<td>217.21</td>
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<td></td>
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Data analyses

Graphs were prepared in Sigmaplot (2000 for Windows, ver. 6.00). Statistical analyses were performed in R ver. 2.11.1 (R Development Core Team, 2010). Stress effects (Figs. 4, 5 and 6) were analysed in a linear mixed model approach (lmer, lme4 package). Probabilities of significant difference from untreated controls were given as $P > |t|$ in a MCMC algorithm. Statistical analyses of linear correlations between concentrations of
individual and total AN-c, and GT data were analysed by a linear regression model approach, recording the slope of the linear model, and probability of significance of the slope being different from 0. Mean values of GT parameters per leaf position of individual plants were used for calculations. Proportion data were calculated as concentration of individual AN-c divided by total concentration of AN-c. In order to achieve homogeneity of variance, the proportion data were transformed by arcsin(√x). When evaluating relations between GT parameters AN-c concentrations and proportions, GT parameters were collected from a separate leaf within the region of leaves collected for the analyses of AN-c (Fig. 3), assuming a relative comparability between these. As statistical modelling showed low loadings of variance from treatments as compared to loadings from the GT parameters, the analysed dataset was a combination of all plants of clones 1 and 2, disregarding treatments. Further certainty for this choice was reached by linear models of individual and total AN-c data versus individual GT data, which irrespective of treatments, showed very similar results to the presented (Table 2). The plants from clones 1 and 2 were evaluated as a combined dataset and formed the basis for conclusions of the controlled experiment. Plants from mildew infected clone 3 were evaluated separately, and formed the basis for conclusions on the effect of the mildew infection. In analyses of individual and total AN-c data from clone 3 in separate linear mixed models, the contribution of variance from the stress treatments proved to be considerably lower than the contribution of variance from mildew infection levels. Therefore the effect of treatments was treated as a systematic factor along with block- and subplot effects in the models used to evaluate the effect of mildew infections in a dataset combined of all clone 3 plants.

Results and discussion

Stress effect on concentrations of AN-c

A broad selection of stress types was applied to provoke a possible reaction in the GT based defence systems of A. annua. In the young upper leaves, stress treatments had no significant effect on the concentration of any of the individual compounds or their total, as compared to controls (Figs. 4 and 6A). This led to rejection of the hypothesis that stress increased the concentration of individual or total AN-c in young upper leaves. In the fully developed lower leaves, the total concentration of all analysed compounds was lower in all treatments compared with the control, although only significantly lower in the sandblasted plants (Fig. 6A). At the level of individual compounds, similar trends were found for all major compounds (Fig. 4): the concentration of AN was significantly lower when plants were stressed by SB (Fig. 4A); the concentration of DHAA decreased significantly upon treatment with NaCl, H_2O_2, SA, and SB (Fig. 4B); the concentration of AA was significantly lower in all stress treatments (Fig. 4C); the concentration of dihydroartemisinic aldehyde (DHAAA) was significantly lower after treatment with H_2O_2, SA, and SB. This led to rejection of the hypothesis that applied stress would increase the concentration of individual or total AN-c in full grown plants. Several studies have demonstrated that creating growth conditions different from “normal” for the plant can change the concentrations of AN and other AN-c (Liersch et al. 1986; Woerdenbag et al., 1993; Qureshi et al., 2005; Ferreira, 2007; Qian et al., 2007; Chaudhary et al. 2008; Özgüven et al., 2008; Pu et al. 2009; Liu et al., 2009; Mannan et al., 2010; Liu et al., 2010; Lei et al., 2011; Aftab et al., 2010; Maes et al., 2011; Rai et al., 2011; Aftab et al., 2011; Banyai et al., 2011). It is interesting to note that most studies demonstrated an increase in AN
concentrations, as opposed to the status quo or slight decrease we observed. The experimental conditions in the papers referred to were very heterogeneous though, and direct comparison with the present results were difficult. Especially the developmental stage and size of the plants is probably important when considering a stress elicited response of the biosynthetic pathway of AN.

The most comparable study to the present was performed by Maes et al. (2011), who treated relatively young *A. annua* seed grown plants, once per week during five consecutive weeks with jasmonate, gibberellin and cytokinin. Analysing the pooled leaves of entire plants, their results confirmed the existence of two different chemotypes: Type I favouring the AA pathway; and Type II favouring the DHAA pathway (Fig. 1). Jasmonate clearly promoted the respective pathways of both chemotypes, and was further associated with increases in GT densities (Type I, and II) and increases in GT area (Type I). Gibberellin had a decreasing or no effect on the pathways of both chemotypes, and was associated with increases in GT densities (Type I, and I) and decreases in GT area (Type I, and II). Cytokinin had a decreasing or no effect on pathways of both chemotypes, and was associated with decreases in GT densities (Type I, and I) and decreases in GT area (Type I, and II). It was thus demonstrated that the applied phytohormones caused quite different responses in the plants. In the present experiment, the plants were larger, and physiologically older than the ones used in Maes et al. (2011), and all stress types caused consistent responses in all the measured parameters as compared with controls (Figs. 4, 5, and 6). Only the amplitudes varied, though a broad diversity of stress types was applied. This is interesting, compared to the quite diverse responses of young plants found by Maes et al. (2011). It is likely that the internal hormonal balance of the larger plants in the present study were more robust to external influences, and that the presently demonstrated responses were a general stress response, rather than the more specific responses shown by Maes et al. (2011) on younger, more susceptible plants.

**Stress effect on ratios between AN-c**

The proportion of individual compounds, relative to the total concentration of AN-c, was not affected significantly in upper leaves (Fig. 5). In the lower leaves, treatments with NaCl, H$_2$O$_2$, SA, and SB caused significantly higher proportions of AN at the expense of a significant lowering of the proportions of: DHAA (NaCl, H$_2$O$_2$, SA, and SB); AA (H$_2$O$_2$, SA, COS, and SB); and DHAA (H$_2$O$_2$, and SA) (Fig. 5). The hypothesis that the applied stress would shift the proportion between AN-c was not confirmed in the upper leaves, but confirmed for all treatments in the lower leaves (Fig. 5). In the lower leaves, the general trend was, that the proportions of the pathway intermediates DHAA, AA, and DHAAA in treated plants were lower than the controls, whereas AN was higher. This leads to the conclusion that the applied stress forced the conversion of precursors towards AN. This further indicated that the treatments affected the maturation rate of the individual GT to be faster as compared to GT on untreated control plants.
Fig. 4. Concentrations of compounds in control and stress treated plants. Black bars show mean values of compound concentrations in upper leaves and grey bars show lower leaves. Error bars indicate standard error of means. Treatments included NaCl, H$_2$O$_2$, salicylic acid (SA), chitosan oligosaccharide (COS), and sandblasting (SB). Numbers of observations follow treatment names as n(upper leaves)/n(lower leaves). Asterisks indicate probability of difference from control (* P< 0.05, ** P< 0.01 and *** P< 0.001).
Fig. 5. Ratios between concentrations of compounds in control plants and stress treated plants. Black bars show mean values of the proportion of individual compounds of total concentration of AN-c of upper leaves and grey bars show lower leaves, error bars indicate standard error of means. Treatments included NaCl, H₂O₂, salicylic acid (SA), chitosan oligosaccharide (COS), and sandblasting (SB). Numbers of observations follow treatment names as n(upper leaves)/n(lower leaves). Asterisks indicate probability of difference from control (* P< 0.05, ** P< 0.01 and *** P< 0.001).
Fig. 6. Total concentration, density of GT, and area of GT. A shows mean values of the total pool of analysed AN-c. B shows mean values of GT density. C shows mean values of GT area. Black bars show upper leaves and grey bars show lower leaves, error bars indicate standard error of means. Treatments included NaCl, H$_2$O$_2$, salicylic acid (SA), chitosan oligosaccharide (COS), and sandblasting (SB). Numbers of observations follow treatment names as n(upper leaves)/n(lower leaves). Asterisks indicate probability of treatment difference from control (* P< 0.05, ** P< 0.01 and *** P< 0.001).

Relations between AN-c concentrations and GT parameters

Analyses of the relationship between GT density and concentrations of AN-c in upper leaves showed that, the higher the GT density, the higher the content of DHAA, AA, and total concentration of AN-c (Table 2). AN showed no linear relation to GT density in upper leaves. There was a weak negative correlation in the still developing upper leaves between AN and GT area, width and length. DHAAA and DHAAOH showed weak positive correlations with length of GT. In the lower leaves, the density of maturing GT showed a weak, but significant, positive correlation with concentrations of AN, DHAA, AA, and total AN-c. Size parameters of GT showed relatively strong positive relationships with concentrations of AN, AA, DHAAA, AAA, and total AN-c. On the other hand concentrations of DHAA seemed not to be related to size of GT. Comparing figures of the influence of stress on the total concentrations of AN-c, density of GT, and area of GT (Figs. 6A-C), similarities in trends are observed. The total pool of AN-c seems to be closely linked to the physical number and volume of the available GT. Though the degrees of explanation ($R^2$) were relatively low, this was further supported by positive correlations between the concentration of total compounds and density of GT in both upper leaves and lower leaves (Table 2).
Table 2. Linear modelling of correlations between compounds and properties of glandular trichomes. Transformed values of proportions and concentration values of artemisinin related compounds were analysed against density, area, width and length of GT from both upper leaves and lower leaves. $R^2$ illustrate the degree of fit of the linear model, * indicate the probability of the slope of the linear model being different from 0 (* P< 0.05, ** P< 0.01 and *** P< 0.001), $\theta$ denotes the slope of the LM, and n indicates the number of values above the detection level.

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In un-stressed plants, regardless of different genetic backgrounds, differences in position on plant, and age of plants, similar positive relationships between density of GT and concentration of AN-c has been demonstrated (Zhang et al., 2006; Kapoor et al., 2007; Arsenault et al., 2010; Graham et al., 2010). Though Maes et al. (2011) concluded that, in stress treated plants (see section 3.1.), increases in GT densities were not necessarily accompanied by increased metabolite production, a positive relationship between density of GT and AN-c is here generally expected, as more GT collectively can contain more compounds per leaf and hence lead to a higher concentration.

The size of the subcuticular cavity of the GT might also be expected to influence the production and storage capacity of AN-c in the GT. The hypothesized positive correlations between size parameters of GT and concentrations of compounds were not confirmed for the upper leaves, but in the lower leaves they was confirmed for AN, AA, DHAAA, AAA, and total compounds. Curiously and unexplained, DHAA seemed unrelated to size of GT in lower leaves. The overall tendencies that larger GT can contain more stored compounds run parallel to the trends of higher GT density resulting in higher concentrations.
**Relationship between ratios of AN-c and GT parameters**

The ratios between the different AN-c in relation to the GT parameters provided a picture of the possible influences of GT age, GT density, and GT size on the conversion of precursors to AN, and possible clues to the understanding of the maturation of GT. In upper leaves, the proportion of AN decreased with higher density of GT, whereas the proportion of DHAA increased. Ratios between compounds seemed unrelated to area of GT, apart from a weak correlation between GT length and proportions of DHAAA and DHAAOH. In the lower leaves, density of GT showed no correlation with the proportional composition of AN-c. The larger the GT of lower leaves, the higher were the proportions of AA, and DHAAA, whereas DHAA declined (Table 2).

A higher GT density in developing upper leaves appeared to be accompanied by a lower degree of conversion of the precursor DHAA into AN. This may be due to a small variation in the exact developmental stage at which these leaves were sampled. The upper leaves were still expanding and thus dispersing the existing GT as discussed by Kjaer et al. (2012). Thus, a higher GT density in upper leaves was likely associated with physiologically younger and less dispersed GT and with a lower degree of progression in the conversion of precursors. In the young upper leaves, the size of GT seemed to be generally unrelated to ratios between AN-c, though longer GT appeared to contain a higher proportion of DHAAA, and DHAAOH. The expansion pattern of developing GT is virtually undocumented in literature, but here it is suggested that much of the expansion in physiologically young GT may be caused by the production of other SM than AN-c, and therefore the size of young GT would not be directly related to ratios of AN-c.

The density of older GT in the lower leaves appeared to be unrelated to the ratios between AN-c. In larger GT in lower leaves there were larger proportions of AA and DHAAA, and smaller proportions of DHAA. Kjaer et al. (2012) suggested that the size of GT on older leaves is linked to the maturity of GT, and that maturation caused GT to shrink. This implies that smaller GT on lower leaves should be accompanied by a higher degree of conversion of precursors to AN, but this was not demonstrated by the present study. It seems clear that GT size was somehow related to the composition of AN-c, although no clear conclusions could be confirmed for these relations. In the literature, only very few reports have described the sizes of GT in *A. annua* (see review in Kjaer et al., 2012). Maes et al. (2011) provided a report describing the size of GT in relation to applied stress, but did not relate this to the content of AN-c. To our knowledge the present study is the first to provide information on the stress induced concentrations and proportions of AN-c as related to sizes of GT in leaves of *A. annua* at two different developmental stages.

At present, no research has focussed on determining the exact volume and content of the subcuticular cavities of individual GT, and thus it is unknown what the ratios between AN-c and additional secondary metabolites are in the subcuticular space. Neither has the relation between the content of the fluid and the physiological age of the GT been demonstrated. Kjaer et al. (2012) showed that various stress treatments did not influence the size of GT on newly developed upper leaves, whereas stressed mature lower leaves possessed significantly smaller GT in relation to controls, and suggested that the stress treatments accelerated the maturation of the GT.
The maturation of GT and the mechanisms of releasing SM to the surroundings are not fully understood. Loss of SM due to collapse of GT, rather than a continuous leaking of AN-c and other SM to the surroundings has been proposed by several researchers (Duke and Paul, 1993; Lommen et al., 2006; Zhang et al., 2006; Arsenault et al., 2010; Kjaer et al., 2012). Though Duke and Paul (1993) pointed out that the preparation method for scanning electron microscopy might provoke additional collapse of GT, Zhang et al. (2006) demonstrated by scanning electron microscopy analyses, that 4% of GT in upper leaves were split open (corresponding to upper leaves in the present study), 12% were split in middle leaves (corresponding to lower leaves in present study), and 25% were split in lower leaves. In the present work, collapsed and thereby empty GT were not registered by the fluorescence microscopy method. The effects of applied stress on densities and sizes of GT were discussed in Kjaer et al. (2012), who suggested that applied stress provoked the maturation process of individual GT, and thereby induced a higher risk of rupturing of the most mature GT. In relation to the above discussion on the concentrations of, and proportions of AN-c, this suggestion implies that the stressed plants might have lost the most mature GT, and thereby potentially masking a pronounced part of the stress response.

**Mildew infected plants**

Many individuals of clone 3 had medium or severe infections of *Golovinimyces* sp. This had a significantly negative effect on the height of plants at the time of initiating the treatments (data not shown). At the time of sampling, heights were no longer significantly different and most plants visibly appeared to have overcome the infection. AN-concentrations in the upper leaves were significantly lower in plants that five weeks earlier had been categorised as severely infected as compared to non-infected, and DHAA and AA showed opposite trends (Figs. 7A-C). The lower leaves in plants rated as medium or severely infected, showed significantly higher concentrations of AN than leaves with no infection, whereas DHAA and AA showed tendencies of decreasing. (Figs. 7A-C). The ratios between AN, DHAA, and AA were relatively unchanged in upper leaves (Figs. 7D-F). Severely infected lower leaves seemingly had a higher degree of conversion to AN, as the proportion of AN was increased at the expense of DHAA (Figs, 7D-F). The total concentrations of AN-c (Fig. 8A) were similar, irrespective of infection levels, in upper leaves, whereas lower leaves showed significantly higher total concentrations with increasing severity of infection. Density of GT and area of GT were not significantly affected by infection levels, though area of GT of the lower leaves tended to be larger at higher infection levels (Figs. 8B-C).

The effect of fungal infections and fungal elicitors on content and proportions of AN-c has, so far, not been demonstrated in full grown *A. annua* plants, but an increasing effect on AN content has been demonstrated in hairy root studies (Liu et al., 1999; Wang et al., 2002; Putalun et al., 2007). Given the substantial difference in concentrations of AN between severely, and non infected plants (Fig. 7A), it is suggested that the AN pathway
Fig. 7. Individual compounds in plants from clone 3 depending on degree of mildew infection. A-C show mean values of compound concentration in plants with different levels of infections. D-E show mean values of proportions of individual compounds of total concentration of artemisinin related compounds. Black bars represent values of upper leaves and grey bars represent lower leaves, error bars indicate standard error of means. Infection levels are followed by n of both upper leaves and lower leaves. Asterisks indicate probability of difference from control (* P< 0.05, ** P< 0.01 and *** P< 0.001).

Fig. 8. Total concentration of compounds, density of GT, and area of GT in plants from clone 3 depending on degree of mildew infection. A shows mean values of total concentration of compound. B shows mean values of densities of GT. C shows mean values of area of GT. Black bars represent values of upper leaves and grey bars represent lower leaves, error bars indicate standard error of means. Infection levels are followed by n of both upper leaves and lower leaves. Asterisks indicate probability of difference from control (* P< 0.05, ** P< 0.01 and *** P< 0.001).
was up regulated by the fungal infection, as it has been demonstrated that phytopathogenic fungi can up regulate the ROS level in plants (Heller and Tudzynski, 2011). Though research has demonstrated that exhaustive extracts of all the compounds from *A. annua* (Soylu et al., 2005; Cavar et al., 2012) and various AN related compounds (Tang et al., 2000; Galal et al., 2005; Gautam et al., 2011) had antifungal activity against several fungi, the knowledge on the interaction between compounds from *A. annua* and fungal attacks is fragmented. The present study demonstrated that further research on the effects of *Golovinomyces* sp. and other fungi on AN would be useful.

**Conclusions**

Repeated stress treatments with NaCl, H$_2$O$_2$, salicylic acid, chitosan oligosaccharide, and sandblasting of full grown *A. annua* did not result in significant changes in the individual or total concentration of AN-c in upper leaves while the lower leaves of sandblasted plants showed significantly lower concentrations of most of the AN related compound (AN-c) and the total AN-c concentrations. The remaining stress types did not result in any significant changes in lower leaves. The ratios between the AN-c were unaffected in upper leaves, and were significantly affected by treatments with NaCl, H$_2$O$_2$ and SA in lower leaves, where the proportion of AN were elevated, while proportions of AA and DHAA were lowered. On lower leaves, densities and sizes of glandular trichomes (GT) were positively related to the total concentrations of AN-c, and concentrations of AN, DHAA, and AA. This indicates that the applied stress types did not magnify the production capacity within the individual GT of a certain size, and that the changes in AN-c concentrations were probably caused by changes in GT numbers and sizes. Density of GT was not related to ratios between AN-c, thus discouraging speculations of GT density affecting a possible source-sink competition between neighbouring GT for metabolites necessary for continuous production. An unintended infection with the powdery mildew *Golovinomyces* sp. in a subset of the plants showed significant increases in the concentration of AN in the GT of lower leaves, as compared to uninfected plants, indicating that the AN pathway was affected by the fungus attack. The overall results suggest that, in agronomic production, it may not be possible to increase the overall yields of AN by applying the demonstrated stress types, though older leaves showed that it may be possible to marginally “force” the conversion of precursors to AN by applying stress. Changes in concentrations of AN-c seemed largely to be explained by changes in GT densities and GT sizes.

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Chapter 8: Paper II


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Chitosan oligosaccharide and salicylic acid up-regulate gene expression differently in relation to the biosynthesis of artemisinin in *Artemisia annua* L.

Heng Yin, Anders Kjaer, Xavier C. Fretté, Yuguang Du, Lars P. Christensen, Martin Jensen, Kai Grevsen


Awaiting publication of special issue.
Short communication
Chitosan oligosaccharide and salicylic acid up-regulate gene expression differently in relation to the biosynthesis of artemisinin in *Artemisia annua* L.

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**ABSTRACT**

*Artemisia annua* L. is a traditional Chinese medicinal plant used for treating fevers and malaria. The primary anti-malarial component is the sesquiterpene lactone peroxide artemisinin, which is accumulated in glandular trichomes. This study investigated the effect of treating plants with chitosan oligosaccharide (COS) and salicylic acid (SA) on both artemisinin production and gene expression related to the biosynthetic pathway of artemisinin. COS up-regulated the transcriptional levels of the genes ADS and TTG1 2.5 fold and 1.8 fold after 48 h individually, whereas SA only up-regulated ADS 2.0 fold after 48 h. LC-MS analysis showed that COS treatment increased artemisinin production in leaves slightly. These results suggest different responses of *Artemisia annua* to COS and SA treatment. The limited effects of COS and SA on artemisinin production reveal that elicitors may not be a universal method for increasing artemisinin yield or the up-regulation takes longer than 48 h to be effective.

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1. Introduction

*Artemisia annua* L., also known as Sweet Wormwood belongs to the plant family Asteraceae, and is an annual medicinal plant, which has been used for traditional Chinese medicine for centuries. The sesquiterpene lactone peroxide artemisinin is the major bioactive compound in *A. annua*, and currently artemisinin-based combination therapy is the most popular treatment method against malaria [1]. In addition to the antimalarial effect, the antioxidant activity of artemisinin and its activity against other parasite species has been demonstrated. A total synthesis of artemisinin was developed in 1980s [2], but the relatively low yield resulting from the synthetic approach has limited its application as an alternative source of artemisinin. Although the precursor to artemisinin, artemisinic acid, can be produced by bioengineering of yeast (*Saccharomyces cerevisiae*) [3], it has not yet been possible to produce artemisinin on an industrial scale by biotechnological methods [4]. The major source of artemisinin is still extraction from plant material [5].

In plants, artemisinin is biosynthesized and stored in specialized anatomical structures, termed glandular trichomes, on leaves, stems and inflorescences [6]. The amount of artemisinin in *A. annua* plants depends primarily on glandular trichome density and biosynthesis capacity in glands; thus the performance of the biosynthetic pathways leading to artemisinin is important to understand. These pathways were thoroughly studied in the 1990s and nearly all the key artemisinin biosynthesis enzymes were identified. In short, the artemisinin biosynthesis starts with the amorpha-4,11-diene synthase (ADS) catalyzing farnesyldiphosphate (FDP) to generate amorpha-4,11-diene. Then cytochrome P450 monoxygenase (*CYP71AV1*) and cytochrome P450 reductase (*CPR*) oxidizes amorpha-4,11-diene to artemisinic alcohol, artemisinic aldehyde, and artemisinic acid sequentially by three steps of oxidation processes. Afterwards, artemisinic acid or its hydrogenated product dihydroartemisinic acid is transformed to artemisinin (Fig. 1) [7]. Later on, some additional genes, which affect the artemisinin biosynthetic pathway, have been identified. For example, a WRKY transcription factor, *ArWRKY7*, has been suggested to regulate the expression of the ADS gene [8].

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\(^{*}\) These two authors contributed equally to this work.
ENHANCER OF GLABRA3 (GL3), which promote trichome initiation were isolated from an A. annua subtractive cDNA library [9]. Due to the large requirement for anti-malarial medicines, there is a substantial interest in methods to improve the content and yield of artemisinin in A. annua. One method of promoting the secondary metabolites production is application of elicitors and plant hormones [10,11]. Chitosan oligosaccharide (COS) is hydrolyzed from chitosan, and is known as an elicitor of plant secondary metabolites production [12]. The effect of COS on artemisinin accumulation in A. annua plant is still unknown. In this study, COS solutions were applied directly to large A. annua plants grown in a greenhouse and the effect of COS on artemisinin production as well as the expression of genes related to its biosynthesis was determined.

Table 1

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2. Materials and methods

2.1. Chemicals

Chitosan oligosaccharides (COS) powder (degree of polymerization 2–10, degree of deacetylation >95%) was obtained from Dalian GlycolBiCo Co., Ltd (Dalian, China); SA was purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Artemisinin (purity >98%) was obtained from My Dinh Extraction plant in Vietnam and Xiang Ji Holley Pharmaceutical Co., Ltd in China. The purity of artemisinin was confirmed by NMR and LC–MS analysis. The organic solvents (chloroform, ethyl acetate, acetonitrile) used in all experiments were of HPLC grade from Fluka Scientific (Slangerup, Denmark). Fumaric acid (FA) (≥98%) was from Sigma–Aldrich (Copenhagen, Denmark).

2.2. Plant material and treatments

From a field grown population of seed propagated A. annua plants (cv. Artemis, F2 seeds, Mediplant, Switzerland), three mother plants was selected for clonal propagation by cuttings, and a sufficient number of plants from these clones was obtained during two multiplication rounds in an environmentally controlled greenhouse. Plants were grown in 3.5L containers in peat moss in a heated greenhouse and drip irrigated twice diurnally with a liquid fertilizer adapted for Asteraceae production. Plants were 14 weeks old and 150–190 cm tall at the beginning of treatment. Leaves of the entire plant were sprayed with aqueous solutions of COS (1 mL L⁻¹, 1 mM) and SA (1 mL L⁻¹, 2 mM) respectively. The experimental design was a randomized complete block design with plots of the three clones (C1, C2, C3) in two blocks. At each sampling time, leaves from four plants from each of the three clones (C1, C2, C3) were collected within each treatment. For the gene expression analyses, the four samples representing individual clones were homogenized into one sample. Only clone C2 was sampled for the artemisinin analysis, and the four samples representing individual treatments were analyzed individually. Leaves from treated and untreated control plants were sampled from predefined positions on the topmost section of the plants at 0 h, 24 h, or 48 h after the treatments, frozen in liquid nitrogen and stored at −80 °C for RNA extraction, or at 48 h and stored at −20 °C for artemisinin extraction.

2.3. RNA isolation and gene expression analysis

Total RNA was isolated from the frozen leaves using Invi-plex lysis reagent (5 Prime GmbH, Hamburg, Germany) according to the manufacturer’s protocol, and the RNA quality was validated using spectrophotometer measurements. For semi-quantitative RT–PCR, first-strand cDNA was prepared with AMV reverse transcriptase (Fermentas GmbH, St. Leon-Rot, Germany) using 1 μg of RNA-treated total RNA as the template in a 10 μl reaction volume with oligo (dt) primer. RT-PCR of the selected genes (ADS, CPR, CYT1A1, TFG1, and WBY1) was performed using the primers listed in Table 1. The housekeeping gene ACTIN was used as control in the experiment. Amplified PCR products (8 μl) were electrophoresed on a 1.5% (w/v) agarose gel and monitored using a Bio-rad mini-transilluminator. The grayscale of each electrophoretic band was quantified by ImageJ software (version 1.37, National Institutes of Health, Bethesda, USA). The expression levels of targeted genes were normalized to those of the ACTIN control gene.

2.4. Artemisinin extraction

Leaf samples were freeze dried and homogenized to fine powder and 400–600 mg of powder was dissolved in 7 mL of dichloromethane and extracted stirred for 24 h at room temperature. Extracts were filtered (Whatman Grade No. 1, 11 μm, 890 mm), and the filter was washed with 2 mL of dichloromethane, the extract was reconstituted with dichloromethane to 10 mL. Solvent was evaporated in vacuo at 40 °C and samples were re-dissolved in 2 mL ethyl acetate. The samples were filtered on PTFE filters (blue 0.2 μm, 017 mm, Le Pha Pack GmbH, Langenwerhe, Germany).
2.5. Artemisinin identification and quantification

Artemisinin was analyzed by LC–MS using a LTQ XL (ESI-2D-iontrap, Thermo Scientific) operated in APCI positive mode and hyphenated with an Accela HPLC Pump and PDA Detector (monitoring at 210, 254 and 360 nm, and scanning from 200 to 600 nm). Settings for the mass spectrometer were 50, 5, and 5 (arbitrary units) for sheath auxiliary, and sweep gas flow rates, respectively. Spray voltage was 5 kV, capillary temperature 450°C, capillary voltage 15 V, tube lens 35 V. Separations were obtained by the solvent gradient A = 0.1% formic acid:FA in H2O, B = 0.1% FA in acetonitrile:isocratic 0–5 min (100% B), linear gradient 5–65 min (1–100% B), isocratic 65–75 min (100% B), linear gradient 75–80 min (100–1% B), isocratic 80–85 min (1% B) on an Eclipse XDB-C18 (5 μm; 250 × 4.6 mm, Agilent). Flow was 500 μL min⁻¹, temperature 60°C, and injection volume 10 μL. Quantification was performed on extracted chromatograms for ion with m/z 283. Quantification range was linear from 29.3 ng mL⁻¹ to 2.588 ng mL⁻¹ (R² = 0.9983).

2.6. Statistics

Analysis of variance was performed on each variable using the SPSS software (IBM, New York, USA). The variations (standard errors, SE), the significances of treatment effects were calculated and tested using the least-significant difference (LSD(0.05)) and Duncan’s multiple range test methods.

3. Results and discussion

Three genes ADS, CPR, CYP (Fig. 1) controls important steps of the artemisinin biosynthetic pathway [7], and were chosen to investigate the effect of SA and COS treatments on the transcription. In the present study, SA treatment significantly up-regulated the ADS expression, but CPR and CYP expression was not affected by SA (Fig. 2). These results suggested that SA treatments affected the early steps of artemisinin production, but had no marked effect on the later steps (Fig. 1). The effect of COS treatment on transcription was stronger than SA in this study, as the expression of ADS was up-regulated significantly and expression of CPR was also slightly promoted (Fig. 3). These results suggest that COS may have a stronger elicitor effect on the biosynthesis of plant secondary metabolites than SA under these conditions.

The expression of ADS, CPR and CYP in response to a number of treatments has been published earlier. But most of these experiments were conducted on suspension or hairy root culture of A. annua and not on large growing plants as in the present study. Published results showed that ADS expression could be up-regulated by SA and methyl jasmonate (MJ), but with no response to DMSO; CPR could be up-regulated by ABA and miconazole, but showed no response to SA and MJ; CYP could be up-regulated by low concentrations of MJ (22 μM), and DMSO, but showed no response to high concentration of MJ (100 μM), SA and miconazole [10,13–16]. The responses in expression of these three genes regulated by SA in our experiments were in agreement with published reports [13,14].

Furthermore, the transcriptional changes of the two transcription factor genes (TTG1, WRKY1) after COS or SA treatments were also investigated in this study (Figs. 2 and 3). These transcription factor genes do not directly take part in the artemisinin synthesis pathway but indirectly regulate the artemisinin production. WRKY1 was suggested to regulate the expression of the ADS gene [8] and TTG1 was reported to promote glandular trichome initiation [9]. The expression of TTG1 was up-regulated by COS treatment revealing the possibility of a positive effect of COS on glandular trichome formation. The expression of WRKY1 was not changed by treatments, suggesting that maybe the induction by COS and SA on ADS was not directly dependent on WRKY1 regulation.

LC–MS analyses showed that the concentration of artemisinin (% w/w in dry leaves) in the elicitor treated plants and in control plants after treatment in 48 h were higher than the control plants at 0 h (start of experiment, 0.111%, 0 h) (Fig. 4). The artemisinin concentration in COS treated A. annua leaves (0.130%, 48 h) was higher than in the leaves of control plants (0.124%, 48 h) and also than SA treated.
plants (0.121%, 48 h) (Fig. 4), but no statistical significant differences were found. The artemisinin content after COS treatment was slightly higher than after SA treatment, which corresponds well with the gene expression results (Figs. 2 and 3). Strangely, the effect of SA treatment is quite limited compared to a reported 50% promotion of the artemisinin concentration compared to controls after 10 mM SA treatment [14]. The effect of COS on large A. annua plants has not previously been reported in literature. In an experiment conducted on hairy root cultures of A. annua, artemisinin production was promoted markedly (6-fold higher than the control) by chitosan (polymer of COS) addition [17]. Whereas, other research reported that chitosan had no effect on artemisinin accumulation in A. annua suspension cultures [18]. So, these reports together with our results suggest that SA and COS treatments function quite differently in different plant cultures. The elicitor effect may depend on many factors such as growth condition, plant variety, growth stage, timing of elicitor treatment, and contact time with elicitor. Finally, the time from elicitor treatment to observed effects on artemisinin production may also play an important role. Therefore, 24 and 48 h after elicitation may have been too short to register any significant changes in artemisinin production despite of the significant promotion in the expression of genes involved in the biosynthesis of this compound.

In conclusion, our experiment showed that COS and SA significantly promoted the expression of some artemisinin synthesis related genes 24 and 48h after application. COS showed a trend of causing a slightly higher artemisinin concentration in leaves. The time frame of 48h may also have been too short to see the full effect of treatment on final artemisinin concentration. The limited effects of COS and SA on concentrations of artemisinin in full grown plants in our study was different from former reports made on non-adult plants [18], and this suggests that signaling molecules may act differently in A. annua depending on the plant model. The use of elicitors therefore may not be a universal method for increasing artemisinin yield from A. annua plants. Our results contribute to elucidate the interactions between different plant elicitors and hormones, their regulation of gene expression and thereby the biosynthesis of artemisinin in large A. annua plants. This may potentially be used in developing applied methods to improve artemisinin production.

Acknowledgements

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References

Short term effects of single versus multiple application of stress on the production of artemisinin and expression of related key genes in large *Artemisia annua* L. plants

Anders Kjær, Heng Yin, Francel Verstappen, Harro Bouwmeester, Elise Ivarsen, Xavier Fretté, Lars P. Christensen, Kai Grevsen, Martin Jensen

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Short term effects of single versus multiple application of stress on the production of artemisinin and expression of related key genes in large Artemisia annua L. plants

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Abstract

• **Aim** The purpose was to apply a range of stress treatments a single time or multiple times to adult plants of Artemisia annua (A. annua) and examine the short term effect on the content of artemisinin (AN) and its immediate precursors, and the expression of the genes ADS, CPR, CYP71AV1, TTG1, and AaWRKY1.

• **Methods** In two separate experiments, large clonally propagated plants were stress treated either once or weekly for five weeks by spraying with salicylic acid, chitosan oligosaccharide, H₂O₂, and NaCl or sandblasting. Contents of AN related compounds (AN-c) were analysed in leaf samples from the upper part of plants using triple-quad UPLC-MRM-MS. Gene expressions were analysed by semi-quantitative RT-PCR.

• **Key Results** Concentrations of the quantified AN-c were unchanged in response to multiple stress treatments of large A. annua plants. Plants responded to a broad range of singularly applied stress treatments by a response wave of temporarily lowering the concentrations of several compounds related to the dihydroartemisinic branch of the biosynthetic pathway of AN. Response waves were similar among treatments, and only the timings varied. Changes in gene expressions were less pronounced after the multiple treatments than after single treatments.

• **Conclusions** Results demonstrated that the applied stress initiated temporary response waves through the DHAA related part of biosynthetic pathway of AN. Results further supported the theory of a temporary build-up of dihydroartemisinic acid hydroperoxide with a relatively fast turnover to AN. Increased activity of ADS genes was reflected by amounts of DHAA, DHAAA, DHAAOH, and AAOH being replenished after the passage of the response wave, whereas AA and AAA were unaffected due to the relative lack of activity of CPR and CYP71AV1. The speed of responses varied, with treatment of SA and COS producing the fastest responses, followed by H₂O₂, NaCl and SB. Differences in compositions of AN-c in control plants between the two experiments, opened for speculations of A. annua using volatile signals to raise the stress level of neighbouring plants.
Keywords: artemisinic acid, artemisinin, dihydroartemisinic acid, hydrogen peroxide, jasmonic acid, metabolites, salicylic acid, sandblasting, stress, triple-quad UPLC-MRM-MS, and wounding.

List of abbreviations:
Artemisinin (AN); Dihydroartemisinic acid (DHAA); Artemisinic acid (AA); Dihydroartemisinic aldehyde (DHAAA); Artemisinic aldehyde (AAA); Dihydroartemisinic alcohol (DHAAOH); Artemisinic alcohol (AAOH); Dihydroartemisinic acid hydroperoxide (DHAAHP); artemisinin related compounds (AN-c); Artemisia annua (A. annua); Salicylic acid (SA); Chitosan oligosaccharide (COS); Sandblasting (SB).

Introduction
The economically important sesquiterpene lactone artemisinin (AN) has only been found naturally occurring in Artemisia annua (A. annua) (Brown, 2010), and the glandular trichomes on the leaves are the primary site of production and storage of this anti-malarial compound and other secondary metabolites with several pharmaceutical and industrial uses (Nguyen et al., 2011). Attempts to synthesize AN or produce AN in engineered yeast and other bioengineered target organisms have proven difficult and until now not economically viable (Ro et al., 2006; Covello, 2008; Graham et al., 2010). So in recent years, a considerable interest has focussed on understanding the natural biosynthetic pathways of AN, the enzymes involved, and the underlying genetic expressions.

If “stress” is defined as conditions which can be considered suboptimal for the plants, the long term effect on content of AN and other AN related compounds (AN-c) has been demonstrated with numerous stress and stress simulating treatments. These include chlormequat, oxidative stress, potassium deficiency, salinity, mycorrhiza, nitrogen application, salicylic acid, jasmonic acid, dimethyl sulphide, cytokinins, wounding, boron, chitosan oligosaccharides, UV-radiation, radioactivity, and gibberellic acid (Liersch et al., 1986; Woerdenbag et al., 1993; Qureshi et al., 2005; Ferreira, 2007; Qian et al., 2007; Chaudhary et al. 2008; Özgöven et al., 2008; Pu et al. 2009; Liu et al., 2009; Mannan et al., 2010; Guo et al., 2010; Liu et al., 2010; Aftab et al., 2010; Lei et al., 2011; Maes et al., 2011; Rai et al., 2011a; Rai et al., 2011b; Aftab et al., 2011a; Aftab et al., 2011b; Banyai et al., 2011, Kjaer et al., 2012b). The results showed that it was possible to affect the concentrations of AN and some of the precursors by stress, often in a positive way, but, to our knowledge, no experiments have till now focussed on the short term effect of stress on the detailed composition of AN and its precursors.

The understanding of the genetic background of the biosynthesis of AN is under continuous elucidation (Arsenault et al., 2010a; Graham et al., 2010; Olofsson et al., 2011), and the majority of the enzymatic and non-enzymatic steps in the pathway are now documented (Bouwmeester et al., 1999; Wallaart et al., 1999; Bertea et al., 2005; Teoh et al., 2006; Liu et al., 2009; Ma et al., 2009; Nguyen et al., 2011; Wang et al., 2011). The proposed biosynthetic pathway (Figure 1) is based on the work of several researchers (Wallaart et al., 2001; Sy and Brown, 2002; Ro et al., 2006; Teoh et al., 2006; Covello et al., 2008; Zhang et al., 2008; Olofsson et al., 2009; Rýden et al., 2010; Polichuk et al., 2010; Maes et al., 2011), and appears to be generally accepted. The most controversial part of the pathway is the possible pathway from artemisinic acid (AA), via arteannuin B and
artemisitene, to AN (Figure 1 (13)). Brown and Sy, 2007 performed a radiolabeling experiment and concluded that no direct path was found from AA to AN. But it was not stated which chemo-type of *A. annua* was investigated, and results are as such not conclusive for all types of *A. annua*. Wallaart et al. (2001) presented two different chemotypes of *A. annua*, Type I, containing relatively high levels of AN and DHAA and low levels of AA, and Type II, containing relatively high levels of AA and low levels of AN and DHAA. Maes et al. (2011), and Wu et al. (2011) demonstrated that a range of phytohormones initiated two different chemotypic expression patterns of key genes involved in the biosynthetic pathway of AN.

![Biosynthetic pathway of AN](image)

Figure 1. Biosynthetic pathway of AN. Names below the compounds with abbreviations used in text given in brackets. Compounds analysed in the present study are underlined. Conversions between molecules are marked with arrows. Commonly accepted conversions between molecules are marked with full arrows, whereas controversial conversions are marked with dotted arrows. Where applicable, the facilitators of the conversions are given next to the conversion number given in brackets.
Similar to the investigation of the effect of stress on the composition of AN-c, several studies have been performed to elucidate the effect of stress and other factors on the expression of genes related to the biosynthetic pathway of AN. These include abscisic acid, jasmonic acid, salicylic acid, gibberellin, cytokinin, dimethyl sulfoxide, sugars, chitosan, arsenic, singlet oxygen, wounding, temperature, position on plant, senescence, and chemotype (Weathers et al., 2006; Jing et al., 2009; Liu et al., 2009; Pu et al., 2009; Zeng et al., 2009; Arsenault et al., 2010; Arsenault et al., 2010; Guo, et al., 2010; Liu et al., 2010; Mannan et al., 2010; Yang et al., 2010; Maes et al., 2011; Wang et al., 2010; Lei et al., 2011; Rai et al., 2011; Olofsson et al., 2011; Zeng et al., 2011; Wu et al., 2011). Results showed that most stress types and other factors affected the gene expressions, though the overall understanding of the mechanisms of stress responses remain fragmented.

The current understanding of the possible stress induced intra- and interplant communication by signal molecules in *A. annua* is currently limited. To our knowledge, only Rapparini et al. (2008) have investigated the possible interplant communication by emission of volatile organic compounds (VOC) in *A. annua*, and only found a few insignificant effects of arbuscular mycorrhizal colonisation. However, in other species of *Artemisia* (*A. tridentate, A. cana,* and *A. douglasiana*) experiments have elucidated that the emissions of VOC play key roles in the defence response to wounding and the subsequent damage induced by herbivores, often to the extent that the emission of VOC play a larger role than the internal signal transport within the individual plants (Karban et al., 2006; Kessler et al., 2006; Shiojiri and Karban, 2006; Shiojiri and Karban, 2008; Shiojiri et al. 2009). Species of *Artemisia* are reportedly very active emitters of a broad spectrum of VOC, including methyl jasmonate, terpenoids and a range of green leaf VOC (Kessler et al., 2006), but the exact mechanisms in interplant communication remain uncertain, though methyl jasmonate seems to play a key role (Preston et al., 2001; Preston et al., 2002; Preston et al., 2004). Curiously the intense interest in the above mentioned *Artemisia* species was founded in the development of cultivation practices in which VOC’s from *Artemisia* plants are used to raise the herbivore resistance in tobacco fields.

There is a very limited knowledge on the how the early changes in gene expressions induced by stress changes the detailed AN-c concentrations. Neither is it known if these early stress responses can be sustained over time if stress treatments are applied in higher frequency over time or the plant signalling mechanisms potentially get saturated as a consequence of adaptation of the plant to a new ‘high stress normal’. The purpose of the present study was to investigate the early effects of selected applied stress treatments to large greenhouse grown *A. annua* plants, and study the different responses of plants treated once or multiple times. The analysed responses included detailed changes in concentrations of AN-c and changes in expression of genes related to the biosynthesis of AN. The following hypotheses were tested for both single and multiple stress treated plants relative to control plants at the same time: Stress will, over time, change the concentration of individual and cumulative AN-c, and stress will, over time, change the expression of genes related to the AN biosynthetic pathway. Furthermore, results from the two separate treatment frequency experiments were related to each other, testing the hypothesis that the two treatment regimens would give different results.
Materials and methods

Introduction

Many parts of the methodologies of the present study coincide with the methodologies utilised in Heng et al. (2011) and Kjaer et al. (2012b), but are included here to provide clarity and to emphasize the points of discrepancies from the former studies.

Plant material

From a field population of seed propagated Artemisia annua (cv. Artemis, F2 seeds, Mediplant, Switzerland), two plants were selected as mother plant for clonal propagation by cuttings in a greenhouse (clones 1 and 2). Tip cuttings were ca. 10 cm long and consisted of 4-5 internodes longer than 1 cm. Cuttings were rooted after 2-3 weeks, and potted in 3.5 L containers with Pindstrup No 2 peat moss (Pindstrup Mosebrug A/S, Pindstrup, Denmark). When plants were 9 weeks from propagation and 80-110 cm in height they were transferred from the nursery to the experimental greenhouse, and plants used in experiment 1 (Ex1) were allowed to acclimatize for 7 days before the onset of the treatments, whereas plants used in experiment 2 (Ex2) were left alone for 28 days before the treatments. Plants were 150-190 cm in heights at samplings. During the experiments, plants were drip irrigated twice diurnally with a liquid fertilizer adapted for Asteraceae. Night temperatures ranged from 8 to 12 °C and day temperatures from 10 to 32 °C.

Experiment

The two experiments were carried out during April-May, 2010 as randomized complete block experimental designs with subsampling, and set up in a greenhouse divided into four separate self-contained compartments. In each compartment, two beds were established with 3x25 plants from each of the two clones. Plants were 50 cm apart and individual beds were separated by 100 cm of gravel. Every second row of three plants were left as guard plants, allowing for 12 treatable subplots of three plants in each bed. In two compartments stress treatments were performed five times at weekly intervals for five weeks (Ex1) and in the remaining two compartments, treatments were performed once (Ex2) 21 days after onset of treatments in Ex1 (Figure 1). Treatments included spraying with NaCl (Salina) in 10 g L\(^{-1}\) aqueous solution, salicylic acid (S7401, Sigma Aldrich) in 1 g L\(^{-1}\) aqueous solutions, hydrogen peroxide (Matas, 10%) at 1.0 %, chitosan oligosaccharide (provided by Dalian Glycobio Ltd, China) in 1.0 g L\(^{-1}\) aqueous solutions, and sandblasting (Badger, Model 260-3, aluminium oxide particles). A manual garden vaporizer (Gardena) delivered 1 ml liquid per spray, and at the beginning of Ex1, 24 sprays covered the majority of a plant in a water film. To compensate for the growth during the five week treatment period, two additional sprays were added each week resulting in 32 sprays at last treatment of Ex1 and the single treatment of Ex2. Similarly, sandblasting was carried out for 2x15 seconds at the onset of the experiment (Ex1) and 2x20 seconds at the last treatments (Ex1 and Ex2). Treatments of both experiments were carried out during the late afternoon to minimize any sun scalding effect from water droplets on the leaf surface. To be able to compare results among different plants, an upper leaf was defined on each plant as the first leaf below the apex on the main stem with internodes longer than 2 cm. Leaf samples for analyses of AN-c consisted of 4 entire main stem leaves collected around the upper leaf (two above and two below), frozen at -20°C, freeze dried for 48 hours, and stored at -20°C until extractions.
Leaf samples for RNA extraction consisted of a section of the upper leaf frozen in liquid nitrogen and stored at -80 °C until extractions. Sampling of leaves in Ex1 for analyses of AN-c was performed at 168h after treatments, and for RNA extraction just before treatments (0h) and at 24h and 48h. Sampling of leaves in Ex2 for analyses of both AN-c and RNA were performed just before treatments (0h) and at 24h, 48h and 168h after treatments, and for RNA extraction just before treatments (0h) and at 24h and 48h (Figure 1). In Ex1, six plants from each treatment regime were sampled from each clone at each sampling time, and in Ex2 four plants were similarly sampled. Due to the fact that part of the experimental plants of Ex2 (clone 2) were utilised for separate AN-c analyses described in Heng et al. (2011), only plants of clone 1 were available for AN-c analyses of the present study. To provide consistency in the comparison between AN-c analyses of Ex1 and Ex2, only results of clone 1 of Ex1 was included in the present study, though results of both clones 1 and 2 were available (Kjaer et al., 2012b). The results of the RNA analyses of plants from Ex2 in the present study were a subset of results previously described in Heng et al. (2011), whereas RNA results from Ex1 have not previously been described. To provide enough samples to perform statistical analyses, RNA results from clones 1 and 2 were included from both Ex1 and Ex2. Samples for AN-c analyses were analysed for each individual plant, whereas samples for RNA extractions were pooled and homogenised in samples comprising all plants form each clone under each treatments regime at each particular sampling time and subsequently homogenised.

**Extractions of artemisinin related compounds**

Approximately 50 mg of dry plant material was homogenized in a mixer mill (MM200; stainless steel ball Ø=7 mm; Retsch, Haan, Germany), accurately weighed, placed in an Eppendorf tube with 1.0 ml MeOH (HPLC grade; Th. Geyer, Germany), and vortexed briefly. The tubes were sonicated for 5 min. and centrifuged at 14000 rpm for 5 min. The supernatant was collected and filtered through a 0.45 µm syringe filter into a brown HPLC glass vial. The pellet was redisolved in 0.5 ml MeOH and the procedure of sonication and centrifuging was repeated. The supernatant was again filtered into the vial, and stored at -20°C. Before measurement the samples were diluted 100 times in 20% acetonitrile (Biosolve, Valkenswaard, The Netherlands) containing 0.1% of formic acid.
Detection and quantification by UPLC-MRM-MS

Targeted analysis of compounds was performed with a Waters Xevo tandem quadrupole mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was obtained on an Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 μm; Waters) by applying a water/acetonitrile gradient to the column, starting from 5% (v/v) acetonitrile in water for 1.25 min and rising to 50% (v/v) acetonitrile in water in 2.35 min, followed by an increase to 90% (v/v) acetonitrile in water in 3.65 min, which was maintained for 0.75 min before returning to 5% acetonitrile in water using a 0.15 min gradient. Finally, the column was equilibrated again for the next injection for 1.85 min using this solvent composition. Operation temperature and flow rate of the column were 50°C and 0.5mL min\(^{-1}\), respectively. Injection volume was 5 μL. The mass spectrometer was operated in positive electrospray ionization mode. Cone and desolvation gas flows were set to 50 and 1000 L h\(^{-1}\), respectively. The capillary voltage was set at 3.0 kV, the source temperature at 150°C, and the desolvation temperature at 650°C. The cone voltage was optimized for the different compounds using the Waters IntelliStart MS Console. Argon was used for fragmentation by collision-induced dissociation in the ScanWave collision cell. Quantification of compounds was done by multiple reaction monitoring (MRM). The optimized settings for MRM measurements are listed in Table 1. Both artemisinin and DHAA were a gift from Dafra Pharma (Belgium). DHAA was used by Chiralix (Nijmegen, the Netherlands) to synthesize the other precursors that were checked by NMR and were more than 98% pure. The reference standards were used for external calibration curves.

Table 1. The optimized settings for UPLC-MRM-MS measurements.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Parent (m/z)</th>
<th>Daughter (m/z)</th>
<th>Cone voltage</th>
<th>Collision voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>219.16</td>
<td>145.08</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>159.09</td>
<td>18</td>
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<tr>
<td></td>
<td></td>
<td>210.2</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>DHAAA</td>
<td>221.16</td>
<td>105.14</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>203.2</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>AAOH</td>
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RNA isolation and gene expression analysis

Total RNA was isolated from the frozen leaves using Isol-RNA lysis reagent (5 Prime GmbH, Hamburg, Germany) according to the manufacturer’s protocol, and the RNA quality was validated using spectrophotometer measurements. For semi-quantitative RT-PCR, first-strand cDNA was prepared with AMV reverse transcriptase (Fermentas GmbH, St. Leon-Rot, Germany) using 1 µg DNase-treated total RNA as the template in a 10 µL reaction volume with oligo (dT) primer. RT-PCR of the selected genes (ADS, CPR, CYP71AV1, TTG1, and WRKY1) was performed using the primers listed in Table 2. The housekeeping gene ACTIN was used as control in the experiment. Amplified PCR products (8 µL) were electrophoresed on a 1.5% (w/v) agarose gel and monitored using a Bio-rad mini trans illuminator. The grayscale of each electrophoretic band was quantified by ImageJ software (version 1.37, National Institutes of Health, Bethesda, USA). The expression levels of targeted genes were normalized to those of the ACTIN control gene. Gene expression was not analysed for sandblasting.

Table 2. The primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’–3’)</th>
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<td>1. ADS-F ADS-R</td>
<td>AATAGGGCAAATGAGGGACA GTGCTTGTATTCGTCTCCCAT</td>
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<tr>
<td>2. CPR-F CPR-R</td>
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<tr>
<td>3. CYP71AV1-F CYP71AV1-R</td>
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<td>4. TTG1-F TTG1-R</td>
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<td>5. WRKY1-F WRKY1-R</td>
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</tr>
<tr>
<td>6. ACTIN-F ACTIN-R</td>
<td>ACCCCTAAAAGCTGTTGA ATACCAGCAGCTTCCATTCC</td>
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</table>

Data analyses

Graphs were prepared in Sigmaplot (2000 for Windows, ver. 6.00). Statistical analyses were performed in R ver. 2.11.1 (R Development Core Team, 2010). Effects of stress treatments on AN-c were analysed in a linear mixed model approach (lmer, lme4 package). Probabilities of significant difference from untreated controls collected at the same time were given for results within Ex1 or Ex2. Mean values of concentrations of AN-c of individual plants were used for calculations. For the gene expression data, probabilities of significant difference from the normalised value at 0h were calculated for results within Ex1 or Ex2. Mean values of expression levels from clones 1 and 2 were used for calculations. All probabilities were given as P >|t| in a MCMC algorithm.
Figure 3. Concentration of compounds AN (A), DHAA (B), AA (C), and DHAAA (D) in control and stress treated plants. Treatments included NaCl, H$_2$O$_2$, salicylic acid (SA), chitosan oligosaccharide (COS), and sandblasting (SB). Obliquely shaded bars show results of Ex1 at 168h. Results of Ex2 are shown as: Black bars (0h), dark grey bars (24h), grey bars (48h) and white bars (168h). Results shown as means (n(Ex1)=12 and n(Ex2)=8), and error bars indicate standard error of means. Asterisks (*) indicate probability of difference from controls sampled at the same time within each experiment (* P< 0.05, ** P< 0.01 and *** P< 0.001).
Figure 4. Concentration of AAA (A), DHAAOH (B), AAOH (C), and cumulative compounds (D) in control and stress treated plants. Treatments included NaCl, H$_2$O$_2$, salicylic acid (SA), chitosan oligosaccharide (COS), and sandblasting (SB). Obliquely shaded bars show results of Ex1 at 168h. Results of Ex2 are shown as: Black bars (0h), dark grey bars (24h), grey bars (48h) and white bars (168h). Results shown as means (n(Ex1)=12 and n(Ex2)=8), and error bars indicate standard error of means. Asterisks (*) indicate probability of difference from controls sampled at the same time within each experiment (* P< 0.05, ** P< 0.01 and *** P< 0.001).
3. Results

3.1. NaCl

The effect of spraying with NaCl once (Ex2) was most pronounced at the end of the sampling period (168h). Concentrations of AN and cumulative concentrations were not affected at any time. DHAA, DHAAA, AAOH and DHAAOH concentrations were significantly lower at 168h, as compared to controls at the same time (Fig 1 and 2). Spraying five times (Ex1) resulted in no significant differences in concentrations of AN-c compared to control plants at 168h (Figs. 3 and 4). Comparing the 168h samples from Ex1 and Ex2 showed that concentrations of DHAAA and DHAAOH were markedly higher after multiple treatments than after a single treatment (Figs. 3 and 4). Neither concentrations of AN nor the concentration of cumulative AN-c were observably different between Ex1 and Ex2. Ex1 did not result in significantly higher expression of any of the genes. Ex2 resulted in significantly higher expression of ADS at 24h, 48h and 168h, whereas expression of CPR,

Figure 5. Relative change of expression of genes between controls (0h) and stress treated plants. Treatments included NaCl, H2O2, salicylic acid (SA), and chitosan oligosaccharide (COS). Results of Ex1 are shown as grey bars, and results of Ex2 are shown as black bars. Results are shown as means (n=2), and error bars indicate standard deviation. Asterisks (*) indicate probability of difference from control values from the same experiment at the same sampling time standardised to the value 1 (* P< 0.05).
CYP71AV1, TTG1, and AaWRKY1 were not changed significantly. It is noteworthy, that for CYP71AV1 and TTG1, multiple treatments resulted in a trend of slightly higher expression than single treatments, whereas for the remaining genes, single treatments resulted in a trend of higher expression as compared to multiple treatments (Fig. 5).

3.2. H$_2$O$_2$

The effect of spraying H$_2$O$_2$ once (Ex2) was most pronounced at the middle of the sampling period (48h) and lesser pronounced at the end (168h). Concentration of AN and cumulative concentrations of AN-c were not affected at any time. DHAA, DHAAA, AAA and DHAAOH concentrations were significantly lower at 48h, whereas at 168h significantly lower concentrations were only observed in DHAA and DHAAA (Figs. 3 and 4). Spraying plants five times (Ex1) did not result in significant differences in concentrations of AN-c compared to control plants sampled at 168h (Figs. 3 and 4). Comparing the 168h samples from Ex1 and Ex2 showed that concentrations of AN, DHAA, DHAAOH, and cumulative AN-c were markedly higher after multiple treatments than after a single treatment (Figs. 1 and 2). Ex2 resulted in significantly higher expression of ADS at 24h, 48h and 168h; CYP71AV1 at 48h and 168h; and TTG1 at 48h and 168h. Ex1 only resulted in significantly higher expression of ADS at 24h. Multiple treatments resulted in non-significantly lower expression than single treatments of all genes at all times (except for AaWRKY1 at 24h) (Fig. 5).

3.3. Salicylic acid

The effect of treating once with SA (Ex2) was most pronounced at the beginning of the sampling period (24h) and lesser pronounced in the middle (48h). Concentration of AN and cumulative concentrations of AN-c were not affected at any time. DHAA, DHAAA, DHAAOH and AAOH concentrations were significantly lower at 24h, whereas at 48h significantly lower concentrations were only observed in DHAAA and DHAAOH (Fig 3 and 4). Spraying plants five times (Ex1) resulted in no significant differences in concentrations of AN-c, compared to control plants sampled at 168h (Figs. 3 and 4). Comparing the 168h samples from Ex1 and Ex2 showed that concentrations of DHAA, DHAAOH, and cumulative AN-c were markedly higher after multiple treatments than after a single treatment (Figs 3 and 4). Ex1 only resulted in significantly higher expression of ADS at 48h and in CYP71AV1 at 24h. Ex2 resulted in significantly higher expression of ADS at 24h, 48h and 168h; CPR at 24h; CYP71AV1 at 24h; and AaWRKY1 at 168h. Multiple treatments resulted in non-significantly lower expression than single treatments of all genes at all times (except CYP71AV1 at 48h) (Fig. 5).

3.4. Chitosan oligosaccharide

The effect of spraying with COS once (Ex2) was most pronounced at the beginning of the sampling period (24h). Concentration of AN and cumulative concentrations of AN-c were not affected at any time. DHAA, DHAAA, DHAAOH and AAOH concentrations were significantly lower at 24h (Fig 3 and 4). Spraying five times (Ex1) resulted in no significant differences in concentrations of AN-c, compared to control plants at 168h (Figs. 3 and 4). Comparing the 168h samples from Ex1 and Ex2 showed that concentrations of DHAAA, and DHAAOH were markedly higher after multiple treatments than after a single treatment (Figs 3 and 4). Ex1 resulted in significantly higher expression of ADS at 24h and 48h; and
TTG1 at 24h, and 48h. Ex2 resulted in significantly higher expression of ADS at 24h, 48h and 168h; CPR at 48h; TTG1 at 24h, 48h and 168h and AaWRKY1 at 24h. Multiple treatments resulted in similar or slightly higher expression of many genes as compared to single treatments (except for CYP71AV1) (Fig. 5).

3.5. Sandblasting

The effect of sandblasting once (Ex2) was most pronounced at the end of the sampling period (168h). Concentration of AN and cumulative concentrations of AN-c were not affected at any time. DHAAOH concentrations were significantly lower at 48h and DHAAA, DHAAAOH, and AAOH at 168h (Fig 1 and 2). Sandblasting plants five times (Ex1) did not result in significant differences in concentrations of AN-c, compared to control plants sampled at 168h (Figs. 3 and 4). Comparing the 168h samples from Ex1 and Ex2 showed that concentrations of DHAAA, DHAAOH, AAOH, and cumulative AN-c were markedly higher after multiple treatments than after a single treatment (Figs. 3 and 4). The gene expression was not evaluated on the sandblasted samples.

4. Discussion

4.1. Short term effect of stress treatments on concentrations of AN-c.

Results demonstrated a relatively consistent type of response to all types of stress within each experiment. In Ex1, none of the treatments resulted in significant differences in concentrations of individual or cumulative AN-c at 168h, as compared to controls. In Ex2, a common pattern in the responses was found, but with different timings among the treatments, suggesting the passage of a wave of responses through the biosynthetic pathway of AN. The suggested wave of responses produced by all treatments included temporarily significant decreases in DHAA, DHAAA, DHAAOH, and AAOH, coinciding with non-significant decreases in the cumulative concentration of AN-c, and non-significant tendencies for a later increase in AN concentrations as compared to control plants. SA and COS caused the fastest initiation of the response wave. H2O2 caused an intermediary fast initiation, and NaCl and SB caused the slowest initiations.

The proposed wave seems to be focussed on the “dihydroartemisinic acid” branch of the biosynthetic pathway (right-hand side of the diagram in fig. 1), whereas the “artemisinic acid” part seems largely unaffected (Figs. 3 and 4). There appears to be a drop in the response pattern, after which the concentrations of the decreased compounds returned to values similar to control values (particularly clear for 24h in COS and 48h in H2O2 (Figs. 3 and 4)). It is interesting to note that all significant changes of the proposed wave showed lower concentrations compared to controls, whereas no compounds showed higher concentrations in response to stress treatments. This, and the temporary non-significantly lower cumulative concentration of all the quantified AN-c (Fig. 4D), indicates a temporary build-up of dihydroartemisinic acid hydroperoxide (DHAAHP)(Fig. 1), which was isolated and identified by Wallaart et al. (1999), and is reported to undergo slow spontaneous autoxidation to artemisinin (Sy and Brown, 2002). Lommen et al. (2006) suggested that DHAAHP accumulated in considerable quantities during maturation of the glandular trichomes, but to our knowledge DHAAHP has seldom been quantified in A. annua plants, neither untreated nor stress treated. Only Wallaart et al., (1999) reported a yield of 40mg DHAAHP per 100g dry leaves corresponding to 0.25%, which relative to the reported
0.5-0.8% content of AN is quite a substantial amount. In the present study, the turnover rate from DHAA over DHAAHP to AN is impossible to quantify directly, but it is noteworthy that for the two fastest response waves (SA and COS), the cumulative concentrations of AN-c were close to having returned to the control values at 168h and the concentrations of AN were increased, though non-significantly. This indicates a relatively fast turnover from DHAA via DHAAHP to AN, though more studies are needed to verify this. In the opposite end of the pathway, at the beginning, the results suggest that relatively early after the passage of the response wave, the concentrations of AN-c were replenished toward the levels of the control, though none of the affected compounds actually reached the control levels during the sampling period.

The experiment of multiple stress treatments (Ex1) only included one sampling for biochemical compounds at 168h, and thus any possible wave pattern through time could not be discerned from the results (Figs. 3 and 4). However, it is noteworthy that none of treatments of Ex1 caused significant changes in any of the compounds, as compared to controls. If plants of Ex1 and Ex2 had responded with similar wave patterns, the late responding treatments (NaCl and SB) of Ex1 would have caused decreases in concentrations of DHAA, DHAAA, DHAAOH, and AAOH. As this was not observed, it is suggested that single versus multiple treatments caused different response patterns (see section 4.3. for further discussion).

The present study appears to be the first to propose the existence of a possible stress induced wave through the biosynthetic pathway of AN by a detailed analyses of AN-c. Some aspects of the study have parallels in previous studies, though. Lei et al. (2011) sprayed COS on 45 days old clonally propagated plants, and collected leaves at an unreported position of the plants. They observed temporary increases in concentrations of AN and DHAA, with optimum concentrations at 48h and 24h, respectively, whereas AA showed no significant changes. Liu et al. (2010) wounded 40 cm tall plants, and collected the middle leaves. They observed small, insignificant rises in concentration of AN in the 4-48 hours after treatments, but these were undetectable again at 72h. Banyai et al. (2011) soil drenched 45 days old wild type plants in a GA\textsubscript{3} solution, and collected leaf samples comparable to the ones collected in the present experiments. They demonstrated a temporary significant rise in AN at 24h and 48h, after which AN declined, but rose significantly again at 14-28 days. In the present study, similar trends of several treatments (NaCl, SA, COS and SB) showing non-significant trends of increasing AN concentrations at varying sampling times were observed (Fig. 2), though no signs were observed of these being of a temporary nature as demonstrated by Lei et al. (2011), Banyai et al. (2011) and Liu et al. (2010). The COS induced increase in DHAA concentrations with optimum at 24h observed by Lei et al. (2011) were not confirmed in this study, which demonstrated a decrease at 48h. This, and the similar observations of the temporary decreasing effect of most treatments on the concentrations of DHAA, DHAAA, DHAAOH, and DHAOH have not previously been reported in literature.

4.2. Short term effect of stress treatments on expression of genes

Results from both experiments (Fig. 5) showed that, the most pronounced differences were found in \textit{ADS} (and less pronounced in \textit{AaWRKY7}), which are part of the early biosynthetic pathway of AN, and in \textit{TTG1}, which is associated with processes related to the initiation
and development of glandular trichomes. CPR and CYP71AV1 are associated with the part of the biosynthesis leading to AA (Fig. 1). The expression patterns of CPR and CYP71AV1 showed modest changes in treated plants compared to controls (Fig. 5), and thus very well reflected the lack of responses to treatments observed in concentrations of AAA and AA in both experiments (except H₂O₂ treated plants of Ex2, which had significantly lower concentrations of AAA at 48h). The elevated activities of ADS in response to particularly the single treatments were expected to increase the concentrations of the early precursors such as AAOH and AAA, or the cumulative concentration of all AN-c. This was not observed (Figs. 3 and 4), and contrary to expectations, many treatments actually significantly reduced the concentrations of AAOH (NaCl, SA, COS, and SB) or AAA (H₂O₂) during the sampling period. Seen in hindsight it might also have been interesting to analyse genes related to the pathways leading to DHAA (i.e. DBR2, and ALDH1), as results indicate that the majority of the responses were detected in this part of the pathway (Figs. 3 and 4). Nevertheless the results of the analysed gene expression were interesting, particularly in the comparison of the differences between single and multiple stress treatments discussed below. The timing of the wave patterns observed in the concentrations of AN-c in Ex2 (section 4.1.) could not readily be related to the gene expression patterns, though future analyses of the DBR2 and ALDH1 genes may show these strong relations.

The expression of stress related genes in A. annua has been investigated intensively in recent years (Weathers et al., 2006 (review); Jing et al., 2009 (abscisic acid); Liu et al., 2009 (jasmonic acid); Pu et al., 2009 (salicylic acid); Zeng et al., 2009 (temperature and position on plant); Arsenault et al., 2010 (sugars); Arsenault et al., 2010 (feedback from AN and AA and position on plant); Guo, et al., 2010 (salicylic acid and methyl jasmonate); Liu et al., 2010 (wounding); Mannan et al., 2010 (DMSO, dimethyl sulfoxide); Yang et al., 2010 (senescence and position on plant); Maes et al., 2011 (Jasmonate, gibberellin, and cytokinin); Wang et al., 2010 (methyl jasmonate); (Lei et al., 2011(chitosan); Rai et al., 2011 (arsenic); Olofsson et al., 2011 (position on plant); Zeng et al., 2011 (singlet oxygen); Wu et al., 2011 (methyl jasmonate and chemotype). The experimental setups, size and age of plants, position of sampling on plants, and the hypotheses tested in these studies varied greatly, and consequently the resulting conclusions varied equally, and searching for common trends proved difficult. However, several of the studies demonstrated wave patterns in the expression of AN related genes, in which the expression levels returned to the initial levels within a few hours or a few days. Of particular interest to the present study were the studies performed by Pu et al., (2009), Guo, et al., (2010), and Lei et al., (2011), as these studies involved the application of the same stress treatments as in the present study. Pu et al. (2009) treated 25-30 cm tall plants with salicylic acid and sampled the third visible leaf under the apex for gene analyses, and observed an 8-fold increase of ADS and a 1.5-fold increase of CYP71AV1 at 8h compared to controls, after which the activity was rapidly normalized. Guo et al. (2010) irrigated 30 days old plantlets with SA mixed water, collected samples from an unreported position on the plant, and observed a 7-fold up-regulation of ADS at 24h, whereas CYP71AV1 and CPR showed no changes in activity compared to controls. Lei et al. (2011) sprayed COS on 45 days old clonally propagated plants, collected leaves at an unreported position of the plants and observed a burst of increased ADS activity at 2h after which it was normalized, and a gradual increased activity of CYP71AV1 with an optimum at 24h. The general trend of observing the highest change in activity in ADS was also demonstrated in the present study, though it is
remarkable that Pu et al. (2009) observed a rapid (after 8h) decline in activity, whereas Guo et al. (2010) and the present study demonstrated increased ADS activity much later (24h and 168h later, respectively). The activities of stress related genes after treatments with NaCl and H₂O₂ have not previously been reported.

Olofsson et al. (2011) investigated 1 meter tall plants, and found that younger leaves, compared to older leaves, had up to several hundred-fold higher expressions of genes associated with AN biosynthesis (i.e. ADS, CYP71AV1, DBR2, and ALDH1). This stresses the importance of exactly defining the position of sampling on the plant in order not to mix leaves with different gene expression patterns. The present study was performed on young leaves collected at a fixed developmental stage. By application of methyl jasmonate, Wu et al. (2011) demonstrated that the two chemotypes of A. annua defined by Waalart et al. (2001) exhibited different gene expressions, as chemotype I up-regulated ADS, CYP, DBR2 and ALDH1, and left activity of CPR unchanged, whereas chemotype II down-regulated ADS, CYP, CPR, DBR2, and up-regulated ALDH1. This and previous observations of the composition of AN-c in older leaves (Kjaer et al., 2012b), leads to the belief that the plant material used in the present study belonged to chemotype I. It would be interesting to know the correct chemotypes of the plant material used in all the published studies, as this apparently has a profound impact on the expression of genes related to AN production (Maes et al., 2011).

4.3. Effect of single versus multiple stress treatments

The experiment elucidating the short term effect of a single stress treatment (Ex2) was performed parallel to the experiment investigating the effect of multiple stress treatments (Ex1), and a comparison between the two experiments was performed. However the experimental setup did not allow for a direct statistical evaluation of the comparison, as it did not include a complete randomisation, and furthermore the sampling times of the two experiments were separated in time by one week (Figure 2). Nevertheless some interesting trends were observed. Firstly, we observed that, in some genes, a single stress treatment resulted in visibly higher increases in gene expression than in plants, which were stress treated multiple times, particularly clear in the gene ADS (Figure 5). Secondly, observing the wave of response in single treatment experiment made us anticipate significant decreases of DHAA pathway related compounds in the slow responding treatments by NaCl and SB in the 168h samples from Ex1, but Ex1 results showed no significant decreases in concentration of any of the individual AN-c in response to treatments (Figures 3 and 4). Taking all reservations into account, we hypothesised that a single stress treatment has a higher impact on the defence apparatus of the plants, than in multiple stressed plants, possibly because the multiple treatments led to an adaptation to the treatments. Adaptation to stress has, to our knowledge, not previously been demonstrated in A. annua, but is a feature commonly found in other plants (Joyce et al., 2003; Shetty, 2004).

Analyses of AN-c of Ex1 (Figs. 3 and 4) demonstrated, that when plants were treated multiple times, all treatments, including controls, showed several-fold higher concentrations of DHA AOH and DHAAA (except SA), and markedly lower concentrations of AA (except NaCl) and AAA (except Control, H₂O₂ and SB) at 168h, as compared to the singularly treated plants of Ex2 at 168h. The interesting part of this observation was that the
untreated control plants from the two experiments exhibited equally large differences as the treated plants. Going through the experimental setups and executions produced no obvious explanations to this enigma. The two experiments were conducted in a greenhouse divided by airproof glass walls into four compartments (blocks), and the results from the two blocks of within each experiment were virtually indistinguishable from each other. The only tangible difference among the experiments, apart from the treatment regime, was that the Ex1 plants were sampled one week later than Ex2, but sampling of leaves for analyses was conducted at the same developmental stage of the shoot in both experiments. This questions the presumption that only direct contact with the treatments created a changed AN-c profile in the plants, and facilitates a suggestion that A. annua might possess a mechanism of communication between individual plants by emission of volatiles. As discussed in the introduction, this has not previously been demonstrated in A. annua, but has been demonstrated in other plant species (Mur et al., 1997; Heil and Ton, 2008; Jansen et al., 2010; Falik et al., 2011; Girón-Calva et al., 2012) and in other species of Artemisia (Preston et al., 2001; Preston et al., 2002; Preston et al., 2004; Karban et al., 2006; Kessler et al., 2006; Shiojiri and Karban, 2006; Shiojiri and Karban, 2008; Shiojiri et al. 2009).

Conclusions

These experiments were the first to demonstrate changes in the detailed composition of most of the precursors involved in the biosynthetic pathway of AN in response to a broad range of stress types. Results indicated that single stress treatments sent a temporary response wave through the dihydroartemisinic acid part of the pathway of AN. The response wave included simultaneous significant temporary lowering of the concentrations of DHAA, DHAAA, DHAAOH, and AAOH, non-significant lowering of the cumulative concentration of AN-c, and non-significant rises in concentrations of AN. The speed of responses varied, with treatment of SA and COS producing the fastest responses, followed by H₂O₂, NaCl and SB. A temporary build-up of dihydroartemisinic acid hydroperoxide is suggested to be part of these changes, but not measured here.

Stress treatments primarily caused increased activity in the TTG1 and ADS related to trichome development and early pathway processes, whereas CPR and CYP71AV1, related to the artemisinic acid part of the pathway, only showed modest changes as compared to controls. This reflected the results of the analyses of concentrations of AN-c well, as amounts of DHAA, DHAAA, DHAAOH, and AAOH were replenished, probably due to the increased activity of ADS, whereas the concentrations of AA and AAA were unaffected due to the relative lack of activity of CPR and CYP71AV1. Comparisons between the applications of single versus multiple stress treatments demonstrated differences in responses, with generally stronger changes in gene expressions after a single treatment compared to smaller changes in expression after five consecutive treatments, which may suggest a mechanism of adaptation to stress. Though results were inconclusive in this respect, it is suggested that multiple treated plants performed a signalling by VOC among plants (including controls), which accumulated a stronger effect of elevated stress responses than among the single treated plants.
Chapter 10: Paper IV

Acknowledgements
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Chapter 10: Paper IV


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Chapter 11: Paper V

11. PAPER V

POSTER

N affects the leaf area and glandular trichomes of *Artemisia annua* L.

Anders Kjær, Willemien JM Lommen, Vincent CF Dupré, Kai Grevsen, Martin Jensen

Poster presented at INTERNATIONAL SYMPOSIUM ON AROMATIC AND MEDICINAL PLANTS, Chiang Mai, Thailand, December 2011.

Project funds have been set aside to prepare content as a “Short Communication” or “Research Letter” intended for publication in a peer reviewed journal.

Section 11.6. provides an expanded discussion of the results.
N affects the leaf area and glandular trichomes of Artemisia annua L.

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Introduction and aims

Artemisia annua (A. annua) produces the antimalarial compound artemisinin in glandular trichomes (GT) on the leaves. GT are only initiated in the early primordial stage of leaf development (Duke & Paul, 1993). The aim was to investigate the effect of different nitrogen applications (N) on field grown A.a. plants. It was hypothesised that higher N would not influence the initiation of new GT, and that the total number of GT per leaf and the area of GT would remain unchanged. Leaves were expected to grow larger at higher N and thus “dilute” the density of GT.

Experiment

A randomized complete block experiment with 4 levels of N in 4 replicate blocks was set up in a field at WUR, The Netherlands in June 2010. Plants were clonally propagated from one plant by tip cuttings. A developing leaf was sampled 25 days after N from 3 plants of each plot (Figure 1). The sampled leaves were presumed to be initiated and elongated after the N application. Numbers and projected areas of GT were analysed from 6 subsections (0.5-1 mm2) of fluorescence microscopy photos of the abaxial leaf side (Figure 2). The total number of GT per leaf was obtained by multiplying the area per leaf by the mean density of GT of the 6 subsections. Statistical comparisons were performed using the HSD Tukey test in R (P<0.05).

Figure 1. Growth effects on A. annua plants 25 days after nitrogen application. Numbers indicate application of inorganic nitrogen (kg/ha). The uppermost main stem leaf below the first internode of 1 cm was sampled for analysis.
Figure 2: Trichomes of A. annua contain autofluorescing essential oils, and are clearly visible objects for analyses. Bar =100 μm.

Results and discussion

Increasing N significantly increased the leaf areas (Figure 3B), but leaves showed constant densities of GT, regardless of N (Figure 3A), and thus the calculated result was a significantly higher total number of GT per leaf (Figure 3C). This indicates that leaves were influenced early in their primordial stage to produce more GT by increased N. The highest N also resulted in significantly larger GT as compared to the two lowest N (Figure 3D), which possibly corresponds to an increased production capacity of the individual GT in this developmental stage. The role of differences between treatments in expansion rates of both leaves and GT areas later in development of the crop remains unknown.

Figure 3 A-D: Effect of four levels of nitrogen application on field grown A. annua. All values are mean values. Error bars indicate Standard Error of Means. Different letters above error bars indicate significant difference between treatments (p<0.05). n=12.
Conclusions

N application on field grown *Artemisia annua* significantly influenced the development of GT on leaves initiated after N. Higher levels of N resulted in larger GT and more GT per leaf in this developmental stage. This indicates that N may be a valuable tool to increase GT numbers per leaf and thereby possibly artemisinin yield in field production of *A. annua*.
12. Discussion of project

12.1. Introduction to discussion

The main results and conclusions of the experiments in the present project are all discussed in detail and related to the existing literature in the Papers I-V. Only exception is the discussion of Paper V, which in its present poster format is relatively short, and therefore I have dedicated section 12.6. of this chapter to present a proper discussion.

In the present discussion, I will primarily present an overview of the results, discussions and conclusions, and relate these to each other and the hypotheses presented in Chapter 6. I will further sum up on the knowledge we have gained from the research, and elaborate on some of the interesting observations, discussions and speculations the experiments have brought about. I will relate the findings to a discussion of the possibilities of using applied stress in improved cultivation strategies, and finally I will propose a number of future experiments, which would provide answers to some of the questions raised by the present project.

12.2. Glandular trichomes in Artemisia annua

The fundamental hypothesis of the project was that it would be possible to provoke A. annua plants to initiate and subsequently build more GT in leaves, if these were provoked at a sufficiently early developmental state. When planning and executing the first experiment (Ex1, Paper I) we leaned on a number of experiments which had shown that relatively modest stress treatments significantly increased GT densities in A. annua and other GT bearing plants (Traw and Bergelsen, 2003; Boughton et al., 2005; Holeski et al., 2007; Gonzales et al., 2008; Maes et al., 2008; Liu et al., 2009). Thus, we expected that a single treatment of the top part of plants with the various stress treatments would elicit a significant increase in GT in the upper leaves, and that the different intensities of the applied stress agents would create differentiated datasets, giving us the opportunity to determine optimum intensities of stress agents. However, no significant increases in GT densities were observed, not even in the plants, which were wounded by cutting or sandblasting multiple times (Figure 3A, Paper I). Consequently, in the next experiment (Ex2, Paper I), we increased the doses and frequencies of treatments, and furthermore included the entire plants in the zone of treatments. Again we found no significant responses in the mean values of GT densities in the upper leaves from the overall experiment (Figure 4A, Paper I), and only in an isolated subset we observed modest, but significant increases in GT in response to high concentrations of NaCl, H$_2$O$_2$, and COS (Figure 5A, Paper I). We attributed this stasis in the density of GT to the advanced age and large size of the plants, and suggested that the plants were less plastic in their response to external influences, and thus difficult to provoke to initiate more GT. Going through the literature of stress influence on GT formation in A. annua, it seems evident that the studies demonstrating the ability of stress agents to increase the density of GT were conducted on relatively young plants (Liersch et al., 1986; Kapoor et al., 2007; Liu et al., 2009; Maes et al., 2011) (Table 1, Thesis), whereas the present study was the first to observe larger plants. Searching the literature for research including comparisons of stress effects on younger relative to older plants of all plant species has been fruitless.
Another factor influencing the provokability of the plants was that the used cultivar ‘Artemis’ was a high-yielding cultivar, which possibly already produced as many (or nearly as many) GT as it was possible under the given growth conditions. The allocation of resources for the building, maintenance, and filling of GT is in constant competition with the allocation of resources to other types of cells and their maintenance. It is very possible that a mechanism of feedback inhibition sets an upper limit for the allocation of resources to the initiation of GT. This implies that the investigated leaves may have run into the “saturation” limit discussed by Maes et al. (2011), and only produced the number of GT they were able to allocate resources to per leaf area unit, and/or were genetically destined to produce. This theory was supported by the results of the nitrogen application experiment (Paper V), as GT densities here were the same across treatments, although leaf areas and calculated GT per leaf were significantly higher under higher N application (Figure 3A, C and D, Paper V). Constant GT densities implies that the number of GT per amount dry matter will be the same, no matter how large the leaves are, but that the total number of GT (and thereby possibly the SM production) will be larger per leaf and subsequently possibly per plant.

The dynamic of “dispersal” of the GT during the expansion of the leaves caused some concern during the project, and may have had an influence on the results, both in present studies and in results reported in other literature. The upper leaves of both Ex1 and Ex2 (Paper I) were not fully expanded at the time of sampling, and were thus in the process of dispersing the existing GT on the leaf blades. This would not be a problem if the expansion process progressed at exactly the same rate among all the plants, but if it did not, some of an observed stress effect on GT densities might be attributed to differences in leaf areas and not necessarily to initiation of new GT. In Ex1 (Paper I) we did observe that some treatments increased the areas of upper leaves (Figure 3C, Paper I). In Ex2 (Paper I) we unfortunately did not measure leaf areas, and thus have no definite conclusions on the effect of possible different leaf expansion rates due to the stress treatments.

The usability of density of GT as a valid indicator of the number of GT in leaves and the comparability between GT density results from different positions on plants and between plants grown under varying conditions was also discussed. Most investigators (Liersch et al., 1986; Lommen et al., 2006; Kapoor et al., 2007; Liu et al., 2009; Graham et al., 2010; Maes et al., 2011; Papers I and II) only counted the GT in small predefined areas of the leaves. However, Lommen et al. (2006) showed that leaves of A. annua continuously expanded during growth until maturation. If we accept that GT are only initiated in the early primordial stages (Duke and Paul, 1993), this implies that leaf expansion must “disperse” the GT, as reported by Werker (2000) in leaves of Ocimum basilicum (Lamiaceae) and discussed by Olsson et al. (2009) in A. annua. Arsenault et al. (2010) applied a method of combining GT densities with the densities of the neighbouring epidermal cells, in an attempt to address the problems with differences in leaf expansion. Lommen et al. (2006) applied a method of assessing the total number of GT on individual leaves by extrapolating the counted areas to the total area of the leaf. But no method has yet encompassed the dilution problem fully. Future GT density experiments should always be backed up by leaf area measurements and number of total trichomes per full leaf, though this would often be difficult due to leaves folding during preparation and the sheer number of GT on the leaves (estimated to be up to 300,000 per leaf by Lommen et al., 2006).
In our field experiment (Ex1, Paper I) GT densities in the fully developed lower leaves of untreated large plants showed no signs of changing over a 5-week period (grey bars in Figure 6A of Paper I). Nor was it possible to increase the GT densities of lower leaves by any of the applied stress types (grey bars in Figure 3A, 4A, and 5A, Paper I) in neither a field (Ex1) nor a greenhouse experiment (Ex2). And in the few cases where the applied stress did increase the density of GT, this only happened in upper leaves, which were not initiated or in a very early primordial stage when the application of treatments was initiated (black bars in figure 5A of Paper I). Thus, the results of the present project did not contradict, and possibly confirmed, the belief that the numbers of GT of the fully developed leaves in *A. annua* were predetermined already at an early developmental stage of the leaves (Duke and Paul, 1993).

In the lower leaves, it was hypothesised that these had already initiated a definite number of GT earlier in their development, and would thus not change GT densities under the influence of stress treatments. However, some of the treatments of Ex2 (Paper I) resulted in significantly lower GT densities compared to controls. This had not previously been reported in the literature of *A. annua*, and a plausible explanation was that some of the GT were lost in some way. As previously described (section 3.5., Thesis), the GT go through a maturation process, and at some point they reach a stage where they become brittle and more prone to provoked rupture or auto-collapse (Duke and Paul, 1993). So it seemed likely, but unresolved, that the stress treatments accelerated the maturation process, and thus provoked the plants to lose more GT by auto-collapse.

Our initial hypotheses regarding the size of the GT on upper leaves was that stress treatments would boost the production of SM, and thus cause the GT to expand. This hypothesis was rejected, as several treatments of Ex2 (Paper I) resulted in significantly reduced size parameters of GT, and the remaining treatments of Ex2 and all the treatments of Ex1 resulted in non-significantly reduced size parameters of GT (Figures 3B, 4B-D, and 5B-D, Paper I). Similarly we hypothesised that the GT of the lower leaves would increase in size. Again the hypothesis was rejected, as almost all treatments of Ex2 resulted in significantly reduced size parameters of GT, and all the treatments of Ex1 resulted in non-significantly reduced size parameters of GT (Figures 3B, 4B-D, and 5B-D, Paper I). This has not previously been reported in the literature of *A. annua*, and looking at the literature on other plant species (e.g. Levin, 1973; Turner et al, 2000; Werker, 2000; Sharma et al., 2003; Wagner et al., 2004), no references of stress causing reduced GT sizes were found. However, the present study is one of the first to use fluorescence microscopy to accurately measure GT sizes on a high number of GT in large plants and relate these to the influence of stress.

Speculating on mechanisms, we hypothesised that the observed “shrinking” of the GT was linked to the hypothesised stress accelerated maturation process, but the underlying mechanistic processes was yet unclear. We considered if the content of the GT was in some way up-concentrated during the maturation process, and thus making the GT appear smaller. We also considered if the smaller GT were due to a possible stress induced change of the proportional production between different groups of compounds in the GT. A possible stress induced change in the demographic composition of the size classes of GT was also considered, as stress might cause an increased risk of auto-rupture of the larger GT, and thus only the smaller GT would remain, causing a lower mean value. Finally we
Chapter 12: Discussion of project

Considered that the “shrinking” of the GT was linked to the subcuticular sac loosening from the secreting cells during maturation (Section 3.4., Thesis), as this would possibly remove some stretching restraints on the sac, which would consequently become more circular in form, and seen from above (as we did in the microscope), this would make the perimeter smaller. However, with the available data, it was not possible to determine which of the above theories was the most likely or correct. We hypothesised that in all the scenarios (with the possible exception of the demographic theory), we would see correlations between decreasing GT densities and sizes, and the state of progression of the synthesis of the precursors of AN to the end molecule AN, as this is assumed to indicate a more mature state. We anticipated that the different stress treatments would cause a set of different GT related responses in the plants, as found by e.g. Maes et al. (2011), but the responses were in fact quite similar across the treatments. However we did observe several cases of the plants responding stronger to higher doses than lower doses of treatments, reassuring us that the responses were indeed caused by the treatments.

In the present project we examined the size of GT (projected area, length and width) in newly unfolded leaves and in older leaves. In all untreated control plants we found that the GT of new leaves were of similar or slightly larger size than the GT of older leaves (control treatments in figures 3B, 4B-D, 5B-D, and 6B of Paper I). This indicates that the rate of filling was relatively fast in the early secretive phase, and that the GT reached a maximum size, which was maintained at least until the stage at which the lower leaves were sampled.

We applied two different fluorescence based microscopy methods. In Ex1 (Paper I) we applied a relatively simple method, and the resulting images (Figure 3A, Thesis) showed the fluorescence from a broad spectrum, including the very dominating light from chlorophyll, and the GT in the pictures were all counted manually and the length of only four GT per picture was measured. In Ex2, Paper I we developed and applied a more refined method using specific short pass filters. With these pictures (Figure 3B, Thesis) it was possible to discriminate the GT from the background, and exclude false positives by size and shape recognition using automated computerised picture analyses. This made it possible to detect many more GT in a manageable timeframe, and furthermore to obtain measurements of the length, width and area of all the individual GT. In comparison we based the conclusions of the size of GT in the first experiment on measurements of the length of approximately 2,600 GT, whereas we in the later experiment were able to include measurements of more than 56,000 GT. In research, various methods have been used to detect and quantify GT in A. annua, including light microscopy (Lommen et al., 2006; Kapoor et al., 2007; Liu, et al., 2009; Arsenault et al., 2010.), fluorescence microscopy (Olsson et al., 2009; Graham et al, 2010; Paper I and II), and scanning electron microscopy (Hu et al., 1993; Zhang et al., 2006; Alejos-Gonzalez et al., 2011; Maes et al., 2011). As discussed in the Paper I, no method seems to be better in terms of accuracy, though the advantage of being able to automatically quantify and measure large numbers of GT in fluorescence microscopy pictures does make this method appealing. The only disadvantage found in the fluorescence microscopy method, was the lack of detection of the remnants of collapsed GT, which Zhang et al. (2006) apparently were able to quantify by scanning electron microscopy.
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12.3. Composition of artemisinin related compounds

In Paper II we examined the effect of stress treatments on the composition of AN-c. We focussed the attention on a set of upper leaves, which were developed during the stress treatments, and a set of lower leaves, which were already developed, when we began treatments. This enabled us to draw direct connections between the GT data (Paper I) and the AN data. Several studies have demonstrated that various stress agents caused elevated levels of AN and other AN-c (Table 1, Thesis and Paper II), and we therefore hypothesised that this would also be the case in our experiment. As we had already shown in Paper I that the GT densities in the upper leaves were primarily unchanged or slightly higher in response to treatments, any increase in AN-c concentrations would necessarily have to mean that each individual GT would be producing more AN-c. However, we found that all the stress treatments left the concentrations of all AN-c, and the total concentrations, unchanged in the upper leaves. Treatments actually decreased the concentrations of the AN related compounds in the lower leaves, particularly clear in the plants treated by sandblasting (Figure 4 and 6A, Paper II). In explanation, we found that the lower concentrations of AN-c in the lower leaves were associated with lower densities of GT and lower size parameters of GT (Figure 6 and Table 2, Paper II). This indicated that each individual GT did not enhance their production of AN-c as a consequence of the stress treatments. Similarly the size of GT was found to co-respond with both the concentrations of many individual AN-c, and the total AN-c concentrations. This
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demonstrated that, in the older leaves, the content of AN-c was linked to the physical number and size of available storage organs in the GT (i.e. size and number of available GT). As discussed in Paper II, this has previously been demonstrated in non-stress treated plants, but this report seems be the first to demonstrate it as a consequence of stress treatments of *A. annua* plants. In the upper leaves, however, the connection between the concentration of AN-c and the total storage capacity was not straight forward. We observed correlations between the densities of GT and the concentrations of DHAA, AA and total AN-c concentrations (but not AN) (Table 2, Paper II). However, although the total concentrations of AN-c in the upper leaves were within the same ranges as in the lower leaves, we did not observe the same trend in the upper leaves (figure 6A, Paper II). Here we only observed a minor negative correlation between size parameters of the GT and concentration of AN, and none for the remaining AN-c and the total concentration of AN-c. We speculated whether this observed difference between the upper and lower leaves might be connected to other factors than the biosynthesis of AN-c and possibly be caused by differences in the speed of synthesis of other SM than AN-c, but this remains to be verified.

There were no differences among the ratios between the different AN-c in the upper leaves, but all treatments (except COS) induced a shift in favour of AN in the lower leaves (Figure 5, Paper II), indicating that the existing GT on the lower leaves of the stressed plants had reached a more mature state. In section 12.2., we discussed whether the apparently stress induced “shrinking” of the GT of lower leaves would be accompanied by a more progressed conversion of precursors to AN. However, the results of the correlation studies between the ratios among AN-c and the size parameters (Table 2, paper II) could not directly be related to any of the proposed theories of why stressing the plants was accompanied with decreases in GT size. Assuming that maturation is accompanied by an increased proportion of AN, a negative correlation between the proportion of AN and the size of GT would have been a clear indication of the maturation of GT causing the GT to shrink, either by deformation (by the loosening of the subcuticular sac) or up-concentration of compounds. However, this was not the case. The theory of stress causing a possible change of the proportional production between different groups of SM (i.e. AN-c versus other terpenes, flavonoids, etc.) in the GT was impossible to test with the present data. However, analyses of correlation with the control plants alone (data not shown) and plants of the total experiment (Table 2, Paper II) both showed positive correlations between concentrations of AN-c, on both the individual and total level, thus discouraging this theory. This leaves us with the “demographic model”, in which we hypothesised that stress did promote the maturation process, and that this caused all GT on the lower leaves of the stressed plants to become more prone to auto-collapse, and that the larger GT would auto-collapse more often than the smaller GT, thus causing the mean values to decline. Alternatively the answer lies in a combination of more than one of the theories, or possibly in theories which we did not come up with. We cannot present a clear answer. As Tissier (2012) stated, no research in any GT bearing plant has till now presented an all-inclusive model in which the development of GT from formation to collapse is modelled and neither has the influence of stress on this process.

We deliberately chose the different stress agents to cover a broad spectrum, so we also anticipated observing differences in the biochemical profiles, but as in the GT analyses (Paper I), we observed that all the stress treatments had similar effects on the biochemical
compounds, and only the amplitudes in responses varied. This was in contrast to the findings of e.g. Maes et al. (2011), who found diverging responses to the applied stress agents (jasmonate, gibberellin, and cytokinin). However, observing that their treated experimental plants were seed grown and substantially smaller in comparison to our clonally propagated large plants, we again came to the conclusion that large plants may be less plastic in response to stress treatments. Possibly, but unresolved, our observations were dominated by a general stress response, rather than specific responses to the specific stress agents.

Going through the literature of stress responses of A. annua to various stress agents (Table 1, Section 5.4., Thesis), both on the formation of additional GT and the AN related biochemical composition, leaves us with a complicated picture of stress responses going in many directions. This is partly due to the complexity of responses to different types of stress agents, environmental changes, etc., but probably particularly due to the diversity of experimental designs, administrations of treatments, specificity of sampling, age of plants, chemotypes of plants, and level of detail in the analyses. From the review of Nguyen et al. (2011), we can conclude that many factors can lead to the formation of new GT in A. annua, but we further state that this is particularly likely to happen in young plants, and probably particularly likely to happen in plants, which initially have a comparatively low GT density. Our experiments demonstrated that large plants of a high yielding cultivar were very reluctant in initiating additional GT in response to stress. The understanding of the effect of stress agents on the AN pathway in specific parts of the plant is hampered by the fact that most studies only report AN values, and that many studies are relatively unspecific in their sampling of leaf material. But it stands clear that the pathway is responsive to a very broad spectrum of stress agents, and that more research is needed to fully comprehend the influence of stress on the details of possible bottlenecks, speeds of conversion, etc. of the different compounds in the pathway. Of particular interest to the present project was the searching for indications in existing research of stress causing an increased rate of maturation and subsequent collapse of the GT, and the possible effect on biochemical composition in large plants. Age related declines in GT densities have only been documented in non-stressed plants (Lommen et al., 2006; Arsenault et al., 2010), but no research has previously documented increasing declines in GT densities on older leaves due to stress treatments.

12.4. Mildew infection

In one experiment (Paper III) we observed that one of the clones was infected with powdery mildew, and we excluded this clone from the analyses described above. Analysing the biochemical data in relation to the degree of infection, we found that, the more infected the lower leaves were, the higher the total concentration of AN-c was, though neither the GT density nor the size of GT were increased (Figure 8, Paper II). This indicated that, in contrast to the uninfected clones, the infection did actually force each individual GT of the lower leaves to produce a higher total concentration of AN-c, and store it in almost the same storage space, as only a small non-significant increase in GT size was observed with higher infection level. The upper leaves showed no increased total concentration of AN-c. At the level of individual compounds, we observed that in the lower leaves, the concentration of AN was strongly increased by the infections (Figure 7A), though not countered by a corresponding decrease in earlier precursors to AN. This
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indicates that the In the upper leaves we observed a curious effect, as infected plants seemed to accumulate DHAA, with a resulting lower concentration of AN. None of these infection related responses has previously been reported in the literature, and our theory is that the infection either triggered a specific anti-fungal response in the plants, or triggered a more general stress response. The observations opens for the possibility of using either live non-pathogenic fungus material or inactivated fungus material to elicit an AN enhancing response in the cultivation of A. annua.

12.5. Short term changes in composition of artemisinin related compounds

In the short term experiment, described in Papers III and IV, we observed a differentiated response to the different treatments, primarily in the experiment, in which the plants were treated only once. Here it was suggested that each stress treatment initiated a wave of response through the biosynthetic pathway, particularly focussed on the part of the pathway leading to DHAA (Figure 1, Thesis). The wave consisted of temporary declines in concentrations of DHAA, DHAAA, DHAAOH and AAOH, after which the concentrations apparently returned to their initial values (Figures 3 and 4, Paper IV). Seemingly the wave stopped or was delayed after DHAA, as no significant changes were detectable in the AN concentrations. Furthermore, the treatments caused non-significant declines in the total concentrations of AN-c, which suggested temporary accumulation of DHAAHP (Figure 2, Thesis), though this compound was not quantified in the present study. Although the resolution of the time line was relatively coarse with only four sampling points (0h, 24h, 48h, and 168h), it was nevertheless possible to distinguish that some treatments initiated the wave sooner than others. The fastest responses were caused by SA and COS, indicating that these stress agents had a direct influence on the pathway. Both SA and COS has previously been demonstrated to elicit a fast response (Pu et al., 2009; Lei et al., 2011). H₂O₂ showed an intermediary fast response, indicating that the applied H₂O₂ contributed directly to the internal ROS wave communication in the plants. The two stress treatments, which in theory should act as external stress agents (NaCl and SB), produced the slowest responses, possibly indicating the initiation of a more complex signal way, compared to the stress agents acting directly on the pathway.

When planning the short-term experiment we chose to focus on genes responsible for the expression of enzymes relatively early in the biosynthetic pathway of AN and genes associated with the initiation of GT. Seen in retrospect it might also have been interesting to include genes responsible for the expression of the enzymes DBR2 and ALDH1 (Figure 1, Thesis), as these facilitate the majority of processes we observed to be influenced by the wave of response described above. Consequently we did not clearly recognise the timing patterns of the suggested response waves in the expression of genes (Figures 3, 4 and 5, Paper IV). Nevertheless the results of the gene expressions improved the understanding of the comparative effect of single versus multiple stress treatments discussed below.

The results of the short-term experiment were divided into separate subsets, which were analysed separately, and presented in the two publications Papers III and IV. Paper III presents a narrow insight into the outcome of the experiment and only presents the effect of COS and SA, and only the concentrations of AN, whereas Paper IV takes all the treatments into account, and includes the analyses of a whole range of AN-c. Nevertheless the parallel nature of the two papers provided the opportunity to compare the two sets of
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independent analyses. The gene analyses of Paper III included data from all three clones, whereas Paper IV only included data from two clones, but from the same data set, and thus it is not surprising to see a full overlap of the gene results. The biosynthetic results were analysed in two separate laboratories, using different extraction methods, reference compounds, and techniques of analyses. Thus, it was reassuring to see the resemblance between the AN results of the two papers, both in the level of concentrations, and in the small, non-significant increases in AN concentrations at 48h (Figure 4, Paper III and 3A, Paper IV).

The experiment elucidating the short term effect of a single stress treatment (Paper III and Ex2, Paper IV), was performed parallel to the experiment investigating the effect of multiple stress (Ex1, Paper I and Paper II), and we performed a comparison of the effect of a single treatment with the effect of multiple stress treatments. However, the experimental setup did not allow for a direct statistical evaluation of the comparison, as it did not include a complete randomisation, and furthermore the sampling times of the two experiments were separated in time by one week (Figure 2, Paper IV). Nevertheless some interesting trends were observed. Firstly, we observed that, in some genes, a single stress treatment resulted in visibly higher increases in gene expression than in multiple stress treated plants, particularly clear in the gene ADS (Figure 5, Paper IV). Secondly, observing the wave of response in the single treatment experiment made us anticipate significant decreases of DHAA pathway related compounds in the slow responding treatments by NaCl and SB in the 168h samples from the multiply treated plants, but here results showed no significant decreases in concentration of any of the individual AN-c in response to treatments (Figures 3 and 4, Paper IV). Taking all reservations of experimental design into account, we suggested that a single stress treatment had a higher impact on the protection apparatus of the plants, than in multiple stressed plants, possibly because the multiple treatments led to an adaptation to treatments or a fatigue in the expression of protection related genes. Adaptation to stress has not previously been described in A. annua, but is a mechanism commonly found in plants (Mittler, 2002; Joyce et al., 2003; Shetty, 2004)

Observing the actual concentrations of AN-c, and not just the changes in relation to controls, we observed that the biochemical profiles of the multiply treated plants looked radically different from those of the single treated plants (Figures 3 and 4, Paper IV). Particularly the DHAAA and DHAAOH concentrations were several-fold higher in the multiply treated plants, but also AN, AAOH and total concentrations showed trends of non significantly higher concentrations in some treatments. Focussing alone on each individual stress treatment, this might have led us to conclude that multiply stress treatments, as opposed to single, affected the plants differently. However, observing that the untreated control plants exhibited equally large differences had us rethink this. The two experiments were conducted in a greenhouse divided by glass walls into four compartments, and each of the experiments was conducted in two of these compartments (blocks). Within each experiment the results from the two blocks were virtually indistinguishable from each other, thus ruling out any significant block effect. Sampling of leaves for analyses was conducted at the same developmental stage of the shoot, thus following the progression of the growth of the plants. The only tangible difference among the experiments, apart from the treatment regimes, was the fact that the multiply treated plants were sampled one week later than the singularly treated plants. Attempting to rule out any plausible explanations left us with the notion that the plants in Ex1 had reacted on a population level, and not just
on the individual level. This has been shown in numerous plant species, including other *Artemisia* species, to be possible by communication by VOC (Section 5.1., Thesis). Though unsubstantiated by an experiment designed to verify this hypothesis, communication by VOC seems to be a plausible explanation to the differences we observed. To our knowledge the relation between single and multiple stress treatments in *A. annua* has not been elucidated prior to this study, and definitely more studies are needed to fully understand the dynamics of GT of *A. annua* responding to different numbers and frequencies of stress events.

### 12.6. Effect of nitrogen application on glandular trichomes in Artemisia annua

In our experiment (Paper V) we investigated the effect of four different N application levels, ranging from 0-400 kg ha\(^{-1}\). Bearing in mind that the soil itself contained a basic level of N, and was fertilised to a standard level of P, K and micronutrients, we were thus able to focus on the effects originating from the N applications. We sampled leaves, which were at the same developmental stage across all the treatments, and which had developed after the transplantation and after the N applications. Results showed that N applications did not affect the density of GT significantly (Figure 3A, Paper V), but that the leaf areas were significantly increased in plants grown at the higher N applications in comparison to plants grown at lower N applications (Figure 3C, Paper V). This indicates that the leaves did not just “disperse” a finite number of GT per leaf (Section 12.2., Thesis), but initiated more GT along with the expected increased expansion of the leaves. This demonstrated that the plants determined the number of differentiated GT at an early developmental stage (Duke and Paul, 1993) on the basis of the nutritional status of the plant, and thus the expected final size of the leaf. It was equally interesting to observe that the high N plants apparently did not overspend on the building of GT, and thus ended up with same GT densities as the low N plants. The contents of AN-c were not quantified in the experiment, but according to the above discussion of correlation between concentration of AN-c and density of GT (Section 12.3., Thesis), this would imply that the concentrations of AN-c in the high N plants most likely was not higher as measured per dry weight, but as measured per plant it would be higher. Interestingly the size of GT increased with higher N application, and in relation to the discussion above about correlation between concentration of AN-c and size of GT (Section 12.3., Thesis), this would indeed imply a higher concentration of AN-c as measured per dry weight, however this remains to be tested.

Paper V is, to our knowledge, the first to investigate the initiation of GT in response to different N applications on leaves at a specific developmental stage. We only investigated a small section of the plants, and thus more research is needed to fully understand the impact of nutrients on the GT morphology. Özgüven et al. (2008) demonstrated that increased N application significantly increased the content of AN in whole crop analyses. Curiously, the content of EO did not increase correspondingly, indicating that, within the existing GT, the plant either down-regulated the EO production relative to AN production, or up regulated the AN production relative to the EO production. In deficiency studies of nitrogen, potassium, phosphorus, and lime Ferreira (2007) demonstrated that the various deficiencies resulted in lower plant mass, but that the AN, DHA, AA concentrations were correspondingly lower (except potassium deficiency, which showed a significantly higher concentration of AN). Davies et al. (2009) similarly demonstrated that plant mass increased with increasing N application. In contrast to Özgüven et al. (2008), Davies et al. (2009)
demonstrated a decline in AN concentration with increased N application. Thus the matter of the effect of N application on AN content remains unresolved. The findings of Paper V, corresponds the best with Ferreira (2007) as we observed that smaller leaves also harboured correspondingly less GT, which in turn means that less plant mass can produce correspondingly less AN.

12.7. Cultivation practices

The overall goal of the present project was to develop knowledge, which can be utilised to create better yields of AN in cultivated A. annua. The hope was that a practice of stress treating large plants would lead to increased yields, but evidently we observed the opposite result. Though our conclusions were restricted to small sections of the plants, it seems likely that the overall result of repeated stress treatments was a loss of GT due to increased collapse rates, and thereby a loss of their biochemical content. However, in some plants the loss of GT was countered by small increases in GT densities in the youngest leaves, though this is not thought to contribute much in the overall yield. Our stress experiments demonstrated that the high yielding variety ‘Artemis’ was relatively conservative in initiation of more GT in response to stress treatments, and that there possibly exists a genetically determined upper barrier to the number of GT per mm$^2$ leaf, as also supported by the N application experiment. This discourages any hope of dramatically increasing the GT densities by stress treatments in this variety. The finding that most stress treatments changed the ratios between AN-c in favour of AN may be utilised in improving the yield. Possibly stress treatments applied shortly before harvest will force the conversion to AN, and if the plants are handled carefully, this will possibly not be countered by an increased collapse rate of GT. This needs to be verified, however. The unintended infection with powdery mildew demonstrated that fungal infections or treatments with deactivated fungi material may have the potential to act as potent elicitors of an increased AN production within each individual GT. The short term experiment demonstrated that single stress treatments initiated differently timed waves of response through the biosynthetic pathway of AN, though did not directly increase the concentration of AN significantly. If a cultivation practise using stress treatments is developed in the future, these differently timings of response are important to know and incorporate in the practise. Also important to incorporate in cultivation with stress, is the hypothesised finding that neighbouring plants apparently show common stress patterns, possibly by emission of VOC. This would in practise mean that the farmer would possibly be able to stress treat the whole farm area by stressing a few plants. Or, if the responsible VOC are identified and extracted, these could be administered to the crop to raise the overall stress level. If the suggested mechanism of mature GT becoming more prone to collapse as a result of aging (Duke and Paul, 1993; Ferreira et al, 1997; Lommen et al., 2006; Zhang et al., 2006; Lommen et al., 2007; Arsenault et al., 2010), or stress treatments (Papers I, II, and IV) is correct, the farmer could use this knowledge to protect the plants from stress and disturbances, and thus avoid loss of AN due to loss of prematurely matured and collapsed GT. In future selection and breeding programs, plants with GT, which are less prone to auto-collapse should be selected, i.e. by having thicker walls in the subcuticular sac, or by having an overall slower maturation rate of the GT. Stress treatments could possibly be utilised in the screening of potential breeding plant for these characteristics.
12.8 Future research

The present project has tested the hypotheses and answered the questions raised in the project description. The execution and analyses of the experiments and the review of contemporary research has, however, revealed a number of blank or under investigated areas in the research of *A. annua*. Below I present some questions which could be raised in future research to improve the understanding of the effect of stress on *A. annua*:

- Can the results and conclusions of our prior experiments be confirmed? Our experiments should be independently repeated to verify the drawn conclusions. To strengthen the conclusions, other cultivars could be included along with ‘Artemis’.
- How does the maturation and collapse of GT progress? A fluorescence microscopy or scanning electron microscopy experiment should be set up to follow the development of individual GT (if possible) or age classes of GT from initiation to collapse. To broaden the number of possible tested hypotheses, a number of stress treatments could be included in the experiment.
- How does the age of the plants influence the responses to external stress? A series of plants of different age classes should be subjected to stress treatments, and leaves of developmentally comparable stages should be sampled and analysed.
- How are the relations between the systemically induced signalling of stress and the signalling by VOC among neighbouring plant in *A. annua*? Experiments similar to those conducted by (Preston et al., 2001; Kessler et al., 2006; Shiojiri and Karban, 2008) on other species of *Artemisia*, could elucidate many aspects of the stress communication in *A. annua*.
- Does fungus infections or the application of inactivated fungus material act as stress elicitors in *A. annua*? Experiments using a range of different fungi (live and dead) should be applied to plants to test hypotheses of changed responses.
13. Conclusions

This thesis is comprised of a series of experiments designed to elucidate the effect of a range of stress treatments on the density and size of GT and on the detailed composition of AN and its immediate precursors. In the following, the hypotheses presented in chapter 6 will be evaluated. It was hypothesised that plants would respond to applied stress by initiating a higher number of GT in upper leaves, which were developed after the onset of stress treatments. However, results showed no or very limited increased GT densities in response to stress. This conservatism was primarily attributed to the theory that large plants were less plastic in response to stress treatments than smaller plant. GT densities of lower leaves, which were already developed at the onset of treatments, were hypothesised to be unresponsive to treatments. However, results showed that all stress types of an experiment of repeated stress treatments resulted in significant or non-significant decreases in GT densities. This was attributed to a theory of stress causing a faster maturation rate of GT, resulting in higher rates of auto-collapse of GT, thus leading to lower GT densities. Sizes of GT in both upper and lower leaves were hypothesised to increase due to increased concentrations of AN-c, when the plants were stress treated. However, the size of the GT in developing upper leaves were relatively unresponsive to stress, whereas the GT of lower leaves decreased in size in response to most types of stress. This was again attributed to the theory that stress would cause a faster maturation of GT. The exact mechanism of how this increased maturation of GT influenced the size of GT remains unresolved.

It was hypothesised that stress treatments would lead to both increased concentrations of AN and total concentrations of AN-c in both the upper and the lower leaves. However, upper leaves showed no response to stress in neither individual concentrations of AN-c, total concentrations of AN-c, nor ratios between AN-c. In the lower leaves, stress, opposite to the hypothesis, caused non-significant or significant declines in concentrations of most individual AN-c and total concentrations of AN-c. These declines were believed to be connected to the increased stress induced maturation rates of GT, leading to lower GT densities of AN-c of lower leaves indicated that stress might force the conversion of precursors toward AN, it remains unknown whether this was just an indirect effect of the theorised increase in GT collapse rate. Density of GT were hypothesised to be correlated with the total concentration of AN-c, and this was verified for both upper and lower leaves, along with the concentrations of AN, AA, and DHAA, though AN concentrations curiously did not appear to be correlated to GT densities of upper leaves. It was concluded that the production of AN-c of each individual GT was relatively stable disregardful of stress treatments, and that stress induced changes in AN-c concentrations were correlated to changes in the number of GT.

In an experiment designed to observe the short term effects of a single stress treatment on the composition of AN-c in upper leaves, it was demonstrated that all stress types caused a suggested wave of response through the AN pathway. The wave consisted of temporary declines in the concentrations of compounds leading to AN along the DHAA branch of the pathway, after which the concentrations apparently returned to their initial levels. The waves were not registered significantly in the concentrations of AN, indicating a suggested temporary build-up of DHAAHP. The different stress types were found to initiate the waves with different timings, with SA and COS causing the fastest responses, H₂O₂ causing an
intermediary fast response, and NaCl and SB causing the slowest initiation of the response waves. A comparison of the composition of AN-c and the gene expression of selected genes in single and multiple stress treated plants indicated that single treated plants were more responsive to stress than multiple treated plants, possibly indicating that multiple treated plants were adapted to the stress treatments. The observation that the control plants of the experiment of multiple treatments possessed a different AN-c composition than the control plants of the single treated experiment, lead to the theory that *A. annua* has the ability to raise the stress level on the population level by emission of VOC. Different levels of N application were hypothesised to cause different GT densities and GT sizes. However, plants responded with the same levels of GT densities in all N treatments, whereas increasing N application caused increasing GT sizes.

In conclusion, we have demonstrated that applying stress to large *A. annua* plant is not likely to result in increased yields of AN, but contrarily may results in loss of compounds due to an increased auto-collapse of GT, though the subtle mechanisms of this remains to be demonstrated.
14. References


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