ROOTS AND HORMONES: SYNERGISTIC CONTROL OF ARTEMISININ PRODUCTION IN *ARTEMISIA ANNUA* L. SHOOTS

By

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Abstract

Artemisinin is a potent antimalarial drug produced in the plant *Artemisia annua*. Earlier reports suggested that the roots play a key role in artemisinin production; however, it was not clear if other factors actually affected production instead of roots. Here the role of roots and two phytohormones, NAA and BAP, were studied to determine what role each plays in artemisinin production in the plant. Rooted *Artemisia annua* shoots produced significantly more artemisinin, arteannuin B, and deoxyartemisinin, the end products in the pathway, than unrooted shoots. Although roots do not seem to affect the levels of precursors, artemisinic acid and dihydroartemisinic acid, or regulate the transcription of the genes in the pathway, rooted plants developed larger trichome sacs suggesting that the accumulation of end products is linked to the expansion of the trichome sac. Unrooted shoots are grown in shooting medium containing higher amount of MS salts, vitamins, sucrose and two potent phytohormones, NAA and BAP. Rooted shoots grown in rooting medium containing either one or both of these hormones showed that NAA increased production of arteannuin B in the young leaves and artemisinin in the mature leaves; in mature leaves, however, arteannuin B was inhibited by NAA. BAP induced production of both the precursors and the end products, except for artemisinin, in the young and/or mature leaves. When rooted shoots with their roots removed were grown in rooting medium containing either one of these hormones, artemisinin was significantly less in cultures grown with BAP while there were no differences in metabolite levels in cultures grown with NAA. Although the importance of roots on the artemisinin biosynthetic pathway cannot be concluded, these results help improve our understanding of artemisinin biosynthesis as may prove useful for improving artemisinin production in field-grown crops.

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1. Trichome + roots + ROS = artemisinin: regulating artemisinin biosynthesis in *Artemisia annua* L.

1.1 Abstract

Artemisinin is a highly effective sesquiterpene lactone therapeutic produced in the plant, *Artemisia annua*. Despite its efficacy against malaria and many other infectious diseases and neoplasms, the drug is in short supply mainly because the plant produces such low levels of the compound. This review updates our current understanding of artemisinin biosynthesis with a special focus on the emerging knowledge of how biosynthesis of the compound is regulated *in planta*.

1.2 Introduction

In 2008, there were an estimated 253 million cases and 863,000 deaths caused by malaria (WHO, 2009). Approximately 85% of the cases and 89% of the deaths occurred in Africa where many countries are undeveloped and poor. While artemisinin is very effective against malaria, it remains too expensive for many people in these countries. Its high cost is due mainly to the high demand for the drug and low production *in planta*; *Artemisia annua* yields are typically less than 1.2% of dry weight (Kindermans et al., 2007). Much work is being done to increase the artemisinin supply including breeding high-yielding *A. annua* strains (Graham et al., 2010), producing transgenic *A. annua*, and engineering the artemisinin biosynthetic pathway into *E. coli*, yeast, and tobacco (Arsenault et al., 2008; Covello, 2008). Artemisinin is not only effective against malaria, but other diseases as well including different types of cancer, schistosomiasis, and some viruses such as hepatitis B (Efferth, 2009). Even though a lot of effort is being put towards increasing artemisinin supply, the biosynthetic pathway is not entirely clear and only recently have studies begun to elucidate its regulation. In this review we update recent earlier reviews (Weathers et al., 2006; Covello et al., 2008), but with a focus on what is currently known about the regulation of artemisinin biosynthesis in *A. annua*.

1.3 Biosynthesis of Artemisinin

Artemisinin (AN; Fig. 1) is a sesquiterpene lactone that stems from three isopentenyl diphosphate (IPP) units and/or its isomer dimethylallyl diphosphate (DMAPP). These 5-carbon

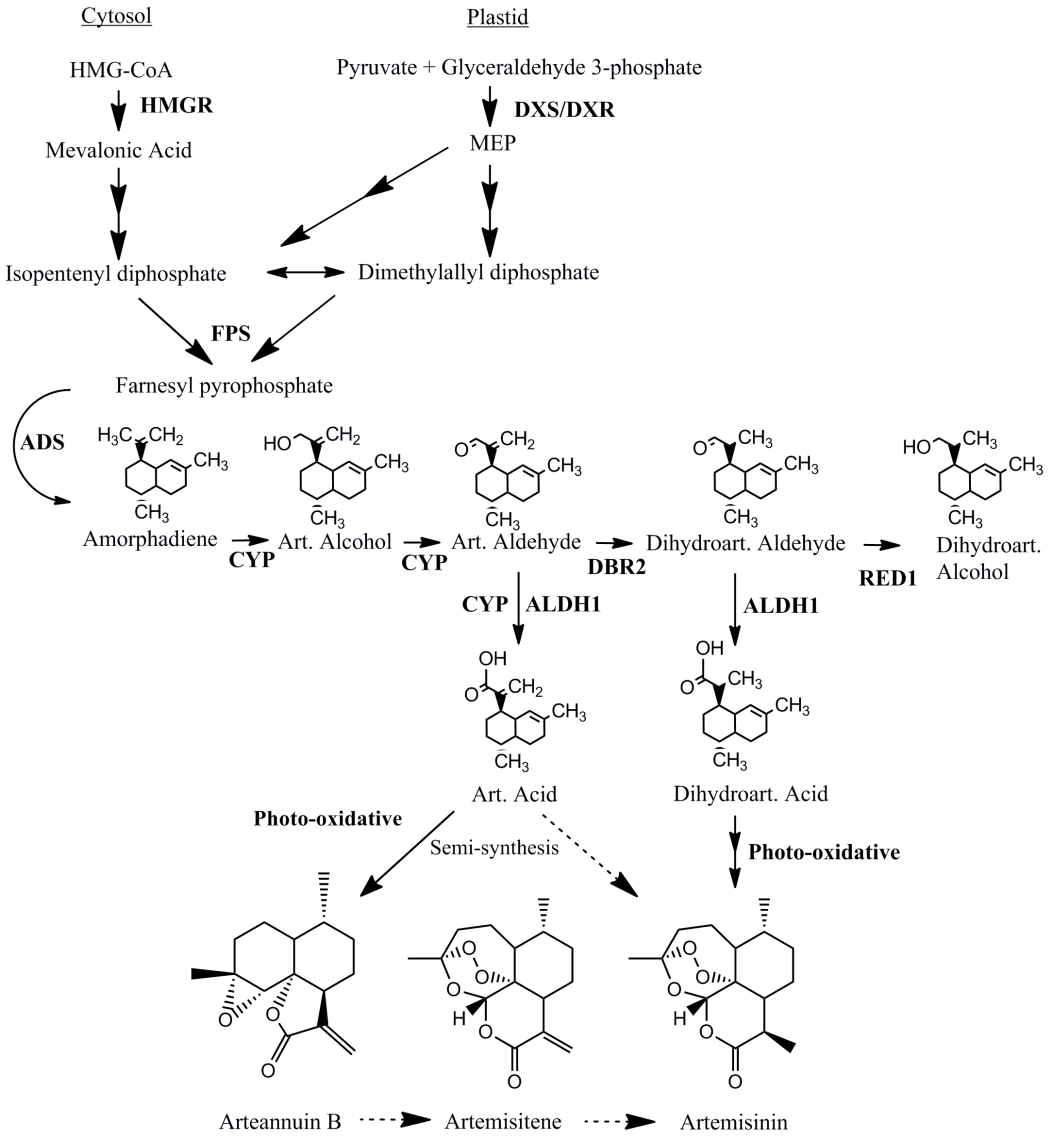


Figure 1.1: Artemisinin biosynthetic pathway. Art Acid, artemisinic acid; ADS, amorphadiene 4,11 synthase; Aldh1, aldehyde dehydrogenase 1; CYP, Cytochrome P 450 CYP71AV1; DBR2, double bond reductase 2; DXR, 1-deoxyxylulouse 5-phosphate reductoisomerase; DXS, 1-deoxyxylulose 5- phosphate synthase; FPS, farnesyl diphosphate synthase; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; MEP, nonmevalonate pathway (plastid pathway); RED1, dihydroartemisinic aldehyde reductase 1.

building blocks of all terpenes can originate from either the cytosolic mevalonate (MVA) pathway or the methylerythritol phosphate (MEP) pathway in the plastid (Fig. 1). In 2007, Towler and Weathers showed that through inhibition of the MVA and/or MEP pathway by mevinolin or fosmidomycin, respectively, AN is produced using IPP/DMAPP from both the

MVA and MEP pathways. This result was quantitatively confirmed by Schramek et al. (2010) using isotopologue profiling of *A. annua* plants with $^{13}CO_2$; the farnesyl diphosphate which eventually yields AN is composed of one IPP/DMAPP from the MEP pathway and two IPP/DMAPP from the MVA pathway.

Although FPP can lead to a number of other compounds in *A. annua* (Weathers et al., 2006), the first step towards artemisinin biosynthesis is the cyclization of FPP to amorpha-4,11-diene by amorphadiene synthase (ADS) (Wallaart et al., 2001; Mercke et al., 2000; Chang et al., 2000). Amorpha-4,11-diene is then oxidized twice by a cytochrome P450, CYP71AV1 (CYP), to form artemisinic alcohol and then artemisinic aldehyde (Teoh et al., 2006; Ro et al., 2006). From here, the exact pathway to artemisinin and other end products are not as clearly understood. It seems that there are two possible routes from artemisinic aldehyde: one route to arteannuin B (AB) and one to AN. The route to AB occurs via oxidation of artemisinic aldehyde to artemisinic acid (AA) either by CYP or an aldehyde dehydrogenase (Aldh1), apparently culminating in AB production (Teoh et al., 2009; Fig. 1). Although Dhingra and Narasu (2001) suggested that AB could be converted to artemisitene (AT) and then to AN in a cell-free system, to our knowledge there has been no other work to validate enzymatic *in planta* conversion of AB to AN via AT. Thus, it is currently thought that AB is one end product of two branches of the AN biosynthetic pathway.

In the other branch of the AN pathway post-CYP, artemisinic aldehyde is converted to dihydroartemisinic aldehyde by the double bond artemisinic aldehyde reductase (DBR2) (Zhang et al., 2008) and then converted to DHAA by Aldh1 (Teoh et al., 2009). DHAA is then converted to AN probably by more than one non-enzymatic, spontaneous photo-oxidation reactions (Wallaart et al., 1999; Brown and Sy, 2007). Recently, Rydén et al. (2010) showed that a new enzyme in the pathway, dihydroartemisinic aldehyde reductase (Red1), appears to be competing for dihydroartemisinic aldehyde, the precursor to DHAA, thereby reducing potential AN production. Once better understood, inhibition of these alternate pathway enzymes offer potential targets for increasing production of AN.

1.4 Morphological and Developmental Regulation of Artemisinin

1.4.1 Trichomes

Trichomes are appendages that develop from the epidermal cells of leaves and flowers and occur in a variety of shapes and sizes: unicellular or multicellular, branched or unbranched, glandular or nonglandular. Glandular trichomes are important to artemisinin biosynthesis because they store many secondary metabolites including AN (Duke et al., 1994; Ferreira and Janick, 1996a).

AN is produced and sequestered in the glandular secretory trichomes (GSTs) of *A. annua* which are composed of 10 cells (Fig. 2): two basal cells, two stalk cells, 4 sub-apical cells, two apical cells, and a subcuticular space where AN accumulates (Ferreira and Janick, 1996a; Olsson et al., 2009). GSTs are located on the leaves and flowers of *A. annua* (Ferreira and Janick, 1996a). Since trichomes are the sites of synthesis of AN, it should be expected that more trichomes would result in more AN produced and indeed there is a strong correlation between AN concentration and trichome density (Kapoor et al., 2007; Graham et al., 2010; Arsenault et al., 2010a). It was also found that younger leaves develop more trichomes and produce more AN than older leaves (Ferreira and Janick, 1996a; Arsenault et al., 2010a). Interestingly, in mature plants, however, senescent leaves provide nearly half of the total AN in the plant (Lommen et al., 2007).

Through microdissection of *A. annua* glandular trichomes and RT-PCR, transcripts of early genes in the AN biosynthesis pathway (*ADS, CYP71AV1, DBR2*) were found only in the apical cells of the trichome, while transcripts of *DXR* from the plastid-localized MEP pathway were only found in the sub-apical cells (Olsson et al., 2009). The apical cells are not green, but the subapical cells are, so the presence of *DXR* transcripts in these latter cells is consistent with functional chloroplasts. Furthermore, transcripts of *FPS* were found in both the apical and sub-apical cells suggesting that the nonplastid MVA pathway is functional in both cell types. It appears that different cells in the glandular trichomes play different roles in the AN pathway. Although no artemisinin was detected in glandless *A. annua*, it is still unclear if any of the genes in the AN pathway are expressed elsewhere in the leaves (Duke et al., 1994).

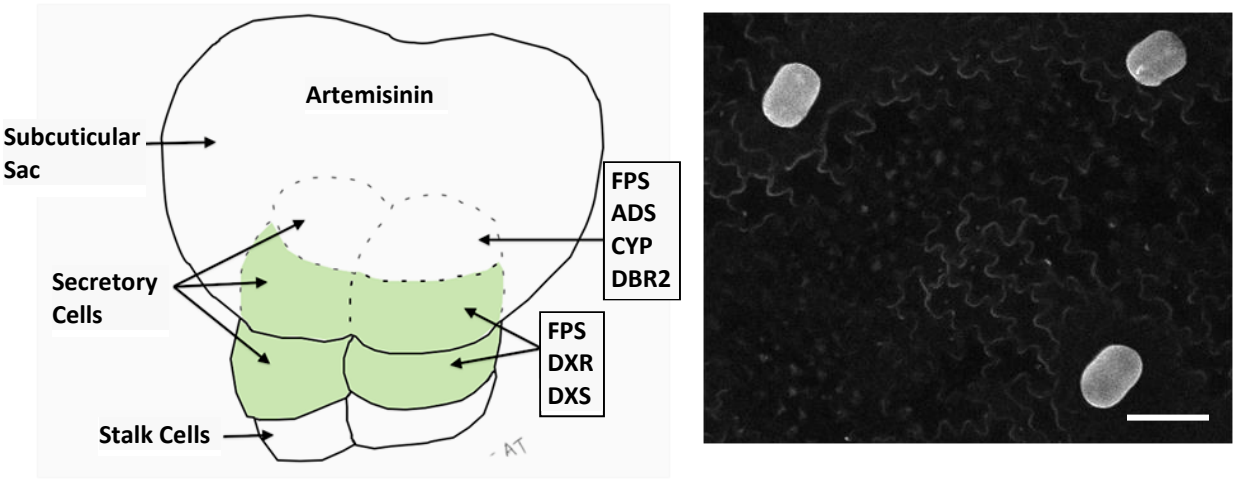


Figure 1.2: Schematic (left) and photo (right) of *Artemisia annua* trichomes. The trichomes on the right autofluoresce when visualized using UV fluorescence microscopy; bar = 100 µm. ADS, amorphadiene 4,11 synthase; CYP, cytochrome P 450 CYP71AV1; DBR2, double bond reductase 2; DXR, 1-deoxyxylulouse 5-phosphate reductoisomerase; DXS, 1-deoxyxylulose 5- phosphate synthase; FPS, farnesyl diphosphate synthase.

Lommen et al. (2006) suggested that upon maturation, trichomes collapse and release AN, which may inhibit the AN biosynthetic pathway. Arsenault et al. (2010a) later tested this hypothesis by spraying the foliage of soil-grown plants during the vegetative stage with either 100 µg/ml AN or AA in 70% ethanol and measuring the mRNA transcripts of *HMGR*, *FPS*, *ADS*, and *CYP*. Compared to the control plants sprayed with only 70% ethanol carrier, plants sprayed with AN only showed decreased transcription of *CYP*. In contrast, plants sprayed with AA showed inhibition of both *ADS* and *CYP*. This suggested that the AN biosynthetic pathway is regulated through feedback inhibition, and that ruptured trichomes may reduce production.

Maes et al. (2010) recently showed that trichome density significantly increased in response to jasmonic acid (JA) and 6-benzylaminopurine (BAP). Seedlings that were typically either high or low AN producers were sprayed and soil dredged with hormones every 2 days for 5 weeks. When they compared trichome size between these two groups of plants, only the low producers showed a significant increase in trichome size. When artemisinic metabolites were measured, however, both the high and the low AN producing plants showed significant increases in AA, AB, DHAA, and AN in response to JA. Overall, however, the largest percent increases in AN and AB occurred in the low AN producing plants. While together these results further

substantiate the relationship between AN production and defense, it is also clear that there may be upper limits to both trichome density as well as AN content.

1.4.2 Roots

In vitro cultured shoots of *A. annua* that develop roots produce more AN than shoots without roots (Ferreira and Janick, 1996b). Similarly, compared to unrooted shoots growing in liquid culture, only rooted shoots of *A. annua* responded to DMSO elicitation of AN production (Mannan et al., 2010). Furthermore, when DMSO was applied only to leaves, there was no change in AN production. Together, these results show that despite the fact that the roots produce neither AN nor its precursors, they play a critical role in AN biosynthesis and possibly in the perception of signals that affect its synthesis. How the roots affect AN production is still unclear, since negligible amounts of transcripts of *ADS* (Kim et al., 2007), *CYP* (Teoh et al., 2006), *DBR2* (Zhang et al., 2008), and *Aldh1* (Teoh et al., 2009) were found in the roots. Only transcripts of a cytochrome P450 reductase apparently associated with CYP activity in *A. annua*, appeared to any significant level in roots (Maes et al., 2010). Clearly more research is needed in order to elucidate how roots increase AN in the shoots of *A. annua.*

1.4.3 Shifting From Vegetative to Reproductive Growth

As *A. annua* plants develop from vegetative to reproductive growth, AN levels increase (Ferrerira et al., 1995; Lommen et al., 2006; Ma et al., 2008). Recently, Arsenault et al. (2010a) measured AN, AB, and their respective precursors, DHAA and AA, as well as transcripts of six genes in the AN biosynthetic pathway (*HMGR, FPS, DXR, DXS, ADS, CYP*) in two types of leaf tissue, flower buds and full flowers in soil-grown *A. annua* in different developmental stages: vegetative, floral budding, and full flowering. When comparing the transcript levels of these six genes in similar leaf tissues at vegetative, budding, and flowering stages, the highest levels of *HMGR, DXR,* and *DXS* were found in leaves at the budding stage and the highest level of *FPS* in leaves at the flowering stage. Although the highest level of *FPS* was coincident with the highest AN level detected, it is unclear how FPS plays a role in AN regulation since overexpression of the FPS gene in *A. annua* has provided conflicting results. While several reported a significant increase in AN when FPS was overexpressed (Chen et al., 2000; Han et al., 2006; Banyai et al., 2010), others reported no change from controls (Ma et al., 2008), or a decrease in AN yield in

some transformants (Banyai et al., 2010). On further analysis, Banyai et al. (2010) observed some gene silencing when two copies of the gene were inserted. In cases where there was a single gene insertion, position of the *A. tumefaciens* delivered transgene was suggested to be the problem. Despite these conflicting results using transgenics, in untransformed *A.annua*, increased expression of FPS under various stimuli generally correlates well with increases in AN production (Arsenault et al. 2010a).

As plants transition to flowering, transcript levels of *ADS* and *CYP* showed a very different pattern than the previously mentioned four genes. Instead of the highest transcript levels of *ADS* and *CYP* appearing during budding or flowering, as one might expect, they were highest during vegetative growth, and then declined significantly during floral development (Arsenault et al., 2010a). Similarly, levels of DHAA were highest in vegetative plants compared to budding and full flower plants. These data suggest that the key metabolic genes responsible for directing terpenoid biosynthesis towards AN may be negatively related to that production or that increased AN negatively regulates these genes *in vivo*. At least during the reproductive stage, when it is the highest, AN appears to inhibit the transcription of *ADS* and *CYP*, which are AN-specific biosynthetic genes. Indeed, this has been confirmed through exogenous application of AN (Arsenault et al., 2010a).

Artemisinin levels are highest either just before or after full flower suggesting that the flowering process may be involved in regulation of AN biosynthesis (Ferreira et al., 1995a; Ma et al., 2008; Arsenault et al., 2010a). This now appears unlikely. Two studies that induced flowering using the early flowering gene CONSTANS (*CO*) (Wang et al., 2004) or the flowering promoting factor1 (*fpf1*) (Wang et al., 2007) showed that although flowering was induced to occur earlier, there was no significant increase in AN levels compared to wild type controls. This suggested that the high level of AN found before or after the flowering stage is not directly linked to the act of flowering.

1.5 Environmental Regulation of Artemisinin

1.5.1 Abiotic

Plants can respond to their environment by altering shape, pigment content, and their secondary metabolites. Not surprisingly, some environmental factors also alter AN production. Lulu et al.

(2008) showed that chilling can affect both AN content and transcript levels of *ADS* and *CYP* and AN levels of *in vitro* cultures. Using real-time RT-PCR and HPLC analysis of AN levels, they observed 11 and 7-fold increases in *ADS* and *CYP*, respectively, after 24 hr in *A. annua* plant cultures chilled to 4ºC for 30 minutes compared to non-chilled cultures. Although chilling also doubled AN levels, higher temperatures (42 ºC), did not seem to affect AN biosynthesis or transcript levels of either *ADS* or *CYP*. Yang et al. (2010) also showed marked increases in *ADS*, *CYP* and *DXS* in response to chilling. In contrast to Lulu et al. (2008) the Yang group also showed a significant increase in both *ADS* and *CYP* in response to a 1 hr dose of UV light.

Drought also affects AN levels in plants (Marchese et al., 2010). Pot-grown plants deprived of water for either 38 or 62 hours showed increased AN leaf concentrations. Only the shorter duration treatment was effective as it did not reduce leaf biomass. Yang et al. (2010) substantiated this response by showing *ADS* increased in shoots when plant roots were allowed to dry for 6 hrs. In contrast, water logging decreased *ADS*, but increased *CYP* transcription; *DBR2* also increased. These results are important for selecting optimal harvest time as there are two benefits: increased AN content overall, and reduced water content of plants thereby reducing drying time.

Various minerals also affect AN yields. For example, boron at limited application levels can increase AN significantly with little or no effect on biomass (Aftab et al., 2010). Levels of H_2O_2 also increased with boron application, so the increase in AN may be a ROS stress response. Phosphate was also shown to increase AN levels, but the mechanism of the stimulus is not known (Kapoor et al., 2007).

1.5.2 Biotic

Although it is likely that pathogens and pathogen related compounds like chitosan are known to be elicitors of secondary metabolites in many plants, only a few reports seem to focus on studies with whole *A. annua* plants. Chitosan is an elicitor of AN production and appears to function by inducing in sequence transcription of the *AaWRKY1* transcription factor that seems to regulate the *ADS* promoter (Ma et al., 2009). In another example, Kapoor et al. (2007) showed that addition of mycorrhizae to roots of potted plants increased AN levels in leaves by as much as 300%, depending on the fungal species, with *Glomus fasciculatum* performing best. This gain

also correlated closely with increased trichome density, a response observed in other species of mycorhizzal plants (Kapoor et al., 2007). In comparison to uninoculated plants, addition of phosphate to plants did not alter AN levels. This study shows that certain biotic factors can play a significant role in altering both the morphology of the plant leaves by increasing trichome numbers, but also that the perception of a stimulating signal again occurs via the roots. Overall there are clearly a number of different environmental effects which are beginning to be elucidated to show how they may alter regulation of the AN biosynthetic pathway.

1.6 Phytohormones and Other Signaling Molecules

1.6.1 GA, ABA, and BAP Effects

Gibberellic acid (GA_3), abscisic acid (ABA), and cytokinins are phytohormones known to affect AN production. GA_3 and 6-benzylaminopurine (BAP) are also known to stimulate trichome development (Maes et al., 2008). When the *A. tumefaciens* cytokinin biosynthetic gene, isopentenyl transferase (*ipt*), was transferred and constitutively expressed in *A. annua*, the *in vivo* content of cytokinins increased along with chlorophyll and AN (Sa et al., 2001) suggesting there is a correlation between AN and cytokinins. This is not surprising since trichome development is known to be stimulated by BAP. Although the level of AN was increased in these transgenic plants, root mass decreased by about 50% indicating that either the source (bacterial gene) or amount of cytokinin produced were problematic.

In a later study Maes et al. (2010) sprayed and soil-drenched two *A. annua* cultivars with either water, 50 μM BAP, or 100 μM GA_3 and saw that both phytohormones stimulated filamentous trichome development, but only BAP stimulated glandular trichome development in both cultivars. Although the two cultivars showed an increase in glandular trichomes, the low artemisinin producing cultivar showed a higher increase than the high artemisinin producing cultivar (Maes et al., 2010). Also, compared to the control, only GA_3 increased the trichome size of the low-artemisinin producing but not the high artemisinin producing cultivar. This suggested that trichomes in the high-artemisinin cultivar were already replete with AN.

ABA plays an important role in a plant's response to different biotic and abiotic stresses; it is also often elevated in response to drought. When soil-grown *A. annua* plants were sprayed with 1-100 μM ABA there was a significant increase in AN content compared to controls (Jing et al.,

2009). There was also an increase in *HMGR*, *FPS*, and *CYP* transcripts in ABA-treated plants after 4, 12, and 8 hours, respectively. This is consistent with the observations of Yang et al. (2010) who reported increased *ADS* expression in plants when roots were subjected to water depletion.

1.6.2 JA and SA Effects

Like many secondary metabolites, AN is thought to play a part in plant defense including against oxidative stress. JA is a secondary messenger that is known to activate a plant's defense system against oxidative stress and to induce production of secondary metabolites (van der Fits and Memelink, 2000). Wang et al. (2009) sprayed soil-grown plants with 300 μM methyl jasmonate and six days after the treatment saw a significant increase in AN compared to controls. Because trichomes are the site of AN biosynthesis and sequestration, Liu et al. (2009) also measured trichome density 14 days after JA treatment. Both trichome density and AN levels increased after JA treatment compared to controls. Later Maes et al. (2010) sprayed and soil-drenched two *A. annua* cultivars with 100 μM JA or water and observed a response similar to that of GA_3 treatment: enhanced trichome density, larger trichomes, and more AN. JA stimulated AN and DHAA production concurrent with increases in *FPS* and *DBR2* in a high artemisinin cultivar. JA also stimulated AB and AA production concurrent with increases in *ALDH1* in the low-artemisinin cultivar (Maes et al., 2010). In addition, JA increased expression of *ADS*, *CYP*, and *CPR* in both cultivars.

Salicylic acid (SA) is another plant signal involved in defense (Durrant and Dong, 2004). When SA was sprayed onto *A. annua* plants, AN, AA and DHAA all increased (Pu et al., 2009). Only *ADS* and *HMGR* showed a measurable increase in transcripts. The ROS molecules, H_2O_2 and O_2^-, both showed significant increases about 4 hrs after treatment and this timing coincided with a significant increase in AN. This study was later confirmed by Guo et al. (2010).

1.7 Other Regulation of Artemisinin

1.7.1 Competing Pathways

Inhibition of squalene synthase (SQS) in other plants has been shown to be co-ordinately regulated with sesquiterpene cyclases. For example, Vögeli and Chappell (1988) showed that in

tobacco when sterols were inhibited, sesquiterpene production increased; conversely inhibition of sesquiterpene synthesis, resulted in enhanced sterol synthesis. In *A. annua*, when SQS was inhibited with miconazole, AN yield increased (Kudakasseril et al., 1987; Towler and Weathers, 2007). Moreover, studies using either an antisense or interference strategy with *A. annua* (Yang et al., 2008), or promoter replacement in *Saccharomyces cerevisiae* (Paradise et al., 2008) showed that when SQS was inhibited, AN metabolites and transcripts of genes in the AN biosynthetic pathway both increased.

1.7.2 Transcription Factors

As mentioned already, a transcription factor, AaWRKY1, was found in *A. annua* and affects AN biosynthesis. AaWRKY1 binds to the W-box cis-acting elements of the ADS promoter and likely regulates ADS gene expression (Ma et al., 2009). WRKY transcription factors are plant specific and bind to the W-box of the promoter of defense-related genes (Ülker and Somissich, 2004).These transcription factors are known to contain a conserved amino acid sequence (WRKYGQK) and a zinc finger-like motif. Using the Plant Cis-acting Regulatory DNA Element (PLACE) database, Ma et al., (2009) found that the *ADS* promoter contains two reverse-oriented TTGACC W-boxes. Subsequently AaWRkY1 was isolated by constructing a cDNA library using mRNA isolated from GSTs and shown to contain the consensus WRKY sequence and a zinc finger-like motif. Ma et al. (2009) showed through an electrophoretic mobility shift assay that AaWRKY1was able to bind to the *ADS* promoter, but unable to bind to a mutated *ADS* promoter. They also showed that an increase in *AaWRKY1* resulted in an increase in *ADS* in transformed *A. annua.* JA also up regulates AaWRKY1, which correlates well with the *ADS* results observed by Maes et al. (2010).

1.7.3 Sugars

Sugar is known to stimulate production of many secondary metabolites, including AN (Wang and Weathers, 2007). When *A. annua* seedlings were inoculated into Gamborg's B5 medium containing 3% (w/v) sucrose, glucose, or fructose for 14 days, glucose stimulated AN production while fructose inhibited production. Increasing the ratio of glucose to fructose showed a direct correlation with increasing AN level (Wang and Weathers, 2007). In a subsequent study, Arsenault et al. (2010b) reported that expression of the genes, *HMGR, FPS, DXS, DXR, ADS,*

and *CYP*, in the AN biosynthetic pathway as well as production of AN metabolites, AN, AB, DHAA, and AA were also affected.

AN was the highest 2 days after inoculation of seedlings into B5 medium containing glucose and as AN increased from day 1 to day 2, DHAA began to decrease (Arsenault et al., 2010b). In medium containing fructose, both AN and DHAA decreased further supporting the earlier observation by Wang and Weathers (2007) that fructose inhibits AN biosynthesis (Arsenault et al., 2010b). On the other hand, AA and AB continued to decline after inoculation into medium containing glucose. In medium containing fructose, however, the AA concentration was always higher than AB. It appeared that AA does not show the same precursor kinetic patterns as was seen with DHAA and AN. Although after 1 day glucose and fructose fed- plants increased *HMGR* transcripts, 3.5 and 2.5, fold respectively, compared to seedlings fed sucrose, *FPS* increased 8 fold, but only in the glucose-fed seedling (Arsenault et al., 2010b). Glucose also increased *DXS* and *DXR* expression in seedlings 1 day after inoculation. After a week's incubation, and compared to fructose-fed seedlings, glucose-fed seedlings showed a 3 fold increase in *ADS* expression relative to sucrose-fed seedlings. *CYP* expression, on the other hand showed a bimodal increase 2 and 7 days after inoculation of seedlings into glucose. Only glucose induced expression of all 6 genes involved in AN biosynthesis. While not practical from the standpoint of production, these studies shown that *in planta* regulation of AN production, at least in young seedlings, is subject to fluctuations in native sugars that are in constant flux in plants.

1.7.4 DMSO

Although AN is thought to be involve in plant defense, how it does this is still unclear. It was suggested that DHAA eliminates reactive oxygen species (ROS) by reacting with it to form AN (Mannan et al., 2010). Staining with 3,3-diaminobenzidine (DAB) for hydrogen peroxide, a common ROS in plants, Mannan et al. (2010) reported an increase in hydrogen peroxide in leaves of 14-day rooted shoots that were exposed to DMSO. The highest amounts of AN were detected in rooted shoots exposed to 0.25% (v/v) and 2% (v/v) DMSO after 7 days. It was also found that AN levels of rooted shoots exposed to 0.25% (v/v) DMSO, the lowest DMSO concentration that can resulted in the highest AN level, were still increasing after 7 days. Interestingly, analysis of DHAA and AA, the precursors of AN and AB, respectively, in the same experiment described above showed that DMSO was able to increase production of

DHAA, but not AA. The highest level of *CYP* transcripts were detected in cultures exposed to 0.5% (v/v) DMSO after 24 hours, but at 0.5% (v/v) DMSO, the AN level was the lowest compared to cultures exposed to 0.1%, 0.25%, 1%, and 2% (v/v) DMSO. There were no significant changes to *ADS* transcripts level in any of the DMSO concentrations.

Ascorbic Acid (vitamin C, AsA) is a ROS scavenger and Mannan et al. (2010) hypothesized that the hydrogen peroxide induced by DMSO should decrease in the presence of AsA. When DMSO-treated plants were incubated with either 10 or 20 mM AsA, levels of peroxide decreased and production of AN significantly decreased by about 80%. This study showed that although DMSO is not part of the natural biochemistry of the plant, it can be a useful tool in helping to elucidate the role of ROS in AN biosynthesis. Indeed ROS, possibly hydrogen peroxide, is playing a role in AN biosynthesis and DHAA is likely acting as a ROS sink with concomitant production of AN.

1.8 Conclusions

Overall a number of different factors are now known to affect the production of AN in *A. annua* and these are summarized in Table 1. Until recently, the one broad rule had been: Trichomes + Roots + ROS = Artemisinin. However, it is now clear that, not surprisingly, phytohormones, in particular jasmonic acid, are playing an important role in regulating production of this terpene. As finer points of control become elucidated, our understanding of terpene production in plants will hopefully enable us to better harness the plant for production of this important therapeutic.

Table 1.1: Summary of known regulating factors affecting artemisinin production in *A. annua.*

	JA	SA	GA_3	BAP	ABA	DMSO	Glucose	Fructose	AA	AN	AM	Chilling	Drought	Water logging*
GENES														
HMGR	nm	+	nm	nm	+	nm	+	+	ns	ns	nm	+	ns	ns
DXS	nm	nm	nm	nm	nm	nm	+	ns	nm	nm	nm	+	+	ns
DXR	nm	nm	nm	nm	nm	nm	+	ns	nm	nm	nm	+	ns	ns
FPS	+	ns	nm	nm	+	nm	+	ns	ns	ns	nm	+	+	ns
ADS	+	+	nm	nm	ns	ns	+	ns	-	ns	nm	+	+	ns
CYP	+	ns	nm	nm	+	+	+	ns	-	-	nm	+	+	ns
DBR2	+	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	ns	ns	+
ALDH1	+	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
METABOLITES														
AA	+	+	ns	ns	nm	ns	-	-	nm	nm	nm	nm	nm	nm
AB	+	nm	ns	ns	nm	ns	-	-	nm	nm	nm	nm	nm	nm
DHAA	+	+	ns	ns	nm	+	-	-	nm	nm	nm	nm	nm	nm
AN	+	+	ns	ns	+	+	+	-	nm	-	+	+	+	nm
Glandular Trichome #	+	nm	ns	+	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
Shoot response to root-perceived signal?	Yes	nm	Yes	Yes	nm	Yes	Yes	Yes	nm	nm	Yes	nm	nm	Yes

*Yang et al. (2010) suggested this induced hypoxia.
nm, not measured; ns, not significant; +, up regulated or increased; -, down regulated or decreased
AA, artemisinic acid; AB, arteannuin B; ABA, abscisic acid; AM, arbuscular mycorrhizae; AN, artemisinin; DMSO, dimethyl sulfoxide; DHAA dihydroartemisinic acid; JA, jasmonic acid; SA, salicylic acid; GA_3, gibberellin 3; BAP, benzyl amino purine.

2. Hypothesis, Objectives, and Organization of Thesis

2.1 Hypothesis and Objectives

Rooted shoots produce more artemisinin than unrooted shoots, so in order to determine how the roots affect the artemisinin biosynthetic pathway, AN metabolite levels and expression of *ads, cyp,* and *dbr2* genes of rooted shoots were compared to expression in unrooted shoots. It is hypothesized that if roots induce higher production of AN, then they should also induce higher production of other artemisinic metabolites in the pathway. Additionally, rooted shoots should have higher expression levels of mRNA transcripts of *ads*, *cyp,* and *dbr2* than unrooted shoots. Since there is a high correlation between trichome population and artemisinin in soil-grown *A. annua*, then it is also hypothesized that rooted shoots, which produce more artemisinin, should have more and/or larger trichomes.

There are two potent phytohormones (NAA and BAP) in the shooting medium that are not present in rooting medium, so it is possible that these hormones may be inhibiting production of artemisinic metabolites. To determine if these two hormones and/or roots play a role in the artemisinin biosynthetic pathway, it is necessary to compare the level of artemisinic metabolites in rooted shoots grown in rooting medium versus rooting medium containing one or both of the phytohormones. If either hormone inhibits artemisinic metabolite production in rooted shoots, then it is possible that the pathway is regulated by hormone(s) and not the roots. On the other hand, these hormones may be down regulating trichome development, which may result in lower artemisinic metabolite production.

2.2 Organization of Thesis

The organization of this thesis is as follows. Chapter 1 is a slightly updated presentation of an already published literature review (Nguyen et al., 2011). The original research reported in Chapter 3 is presented as a draft of a manuscript that will be submitted for publication. Chapter 4 provides an overall conclusion including suggestions for future work.

3. Roots and hormones: synergistic control of artemisinin production in *Artemisia annua* L. shoots

3.1 Introduction

Artemisinin is a sesquiterpene lactone produced in only the glandular trichomes of *Artemisia annua* L. (Duke et al., 1994). It is currently the most effective treatment for malaria and has been found effective against other diseases like schistosomiasis, hepatitis B, and different types of cancer (Efferth 2009; Firestone and Sundar, 2009). The plant is the only economically viable source of artemisinin and studies have generally focused on understanding the biochemistry with an aim to increasing *in planta* production.

The first committed step toward production of artemisinin and the other artemisinic metabolites is the cyclization of farnesyl diphosphate to amorpha-4,11-diene by amorphadiene synthase (ADS; Fig. 3.1). Next, amorpha-4,11-diene is catalyzed to artemisinic alcohol and then artemisinic aldehyde by a cytochrome P450 monooxgenase (CYP71AV1). From here, there are at least two possible routes. The first route is the production of artemisinic acid (AA) from artemisinic aldehyde by either CYP71AV1 or an aldehyde dehydrogenase (ALDH1). Then, AA is converted by photo-oxidation into arteannuin B (AB). The second route leads to production of dihydroartemisinic aldehyde from artemisinic aldehyde in a reaction catalyzed by a double-bond reductase (DBR2). Dihydroartemisinic aldehyde can be reduce to either the less desirable dihydroartemisinic alcohol via dihydroartemisinic aldehyde reductase (RED1), which is not yet well studied *in planta*, or to dihydroartemisinic acid (DHAA) by ALDH1. Finally, DHAA can be converted into either artemisinin (AN) or deoxyartemisinin (deoxyAN) by an apparently nonezymatic photo-oxidation reaction. For purposes of this study, AA and DHAA are considered as “precursors” to AB, and to AN/deoxyAN, respectively. AB, AN, and deoxyAN are the “end products” of these two branches of the artemisinin biosynthetic pathway (Figure 3.1).

Ferreira and Janick (1996b) found that *A. annua* shoots grown in shooting medium produced significantly less AN than shoots grown in rooting medium where they later developed roots. The biggest difference between shooting and rooting medium is that shooting medium contains two

phytohormones, NAA, an auxin, and BAP, a cytokinin. *A. annua* roots also seem to perceive signals that affect AN production in the shoot. Mannan et al. (2010) showed that roots, but not shoots, were able to perceive an elicitation signal from DMSO and produced more AN in their shoots. Although those studies suggested that the roots might be playing a role in the artemisinin biosynthetic pathway, no AN or other artemisinic metabolites were detected in the roots (Woerdenbag et al., 1991; Kim et al., 1992; Ferreira et al., 1995b; Gupta et al., 2002). Furthermore, transcripts of key genes in the pathway are at best barely detectable in the roots (Teoh et al., 2006; Kim et al., 2008; Zhang et al., 2008; Teoh et al., 2009; Olofsson et al., 2011). Taken together those studies suggested that the roots may be playing some regulatory role on shoot production of AN.

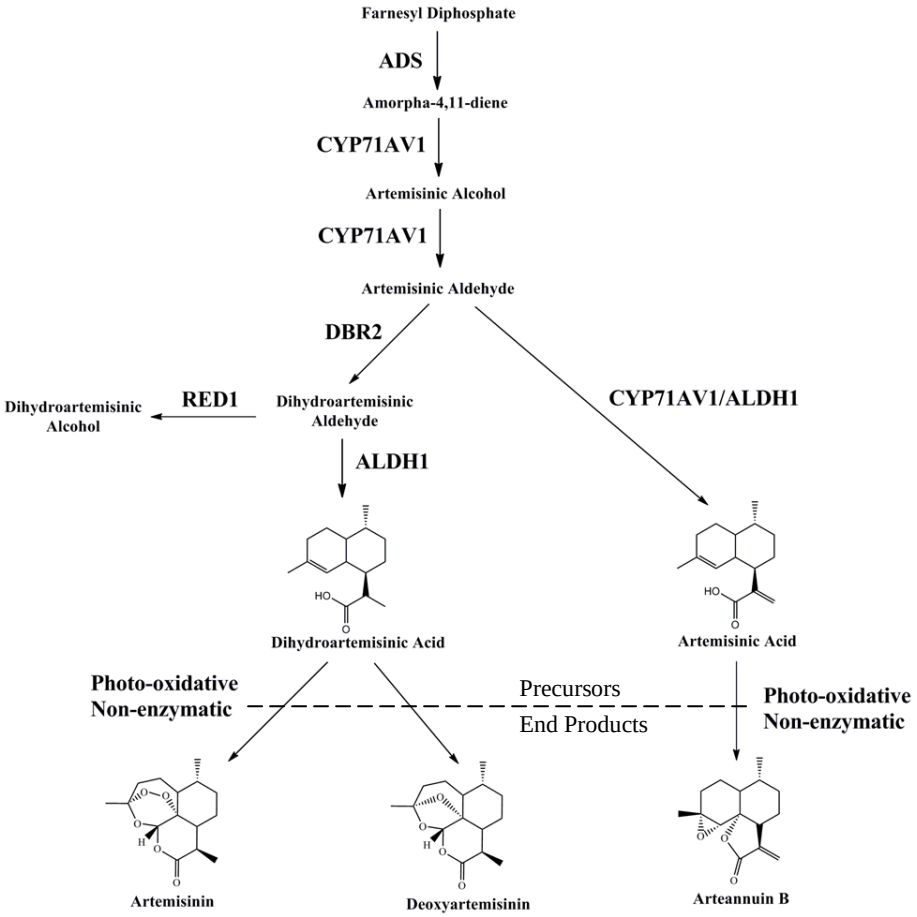


Figure 3.1: Artemisinin biosynthetic pathway. Abbreviations: ADS, amorphadiene synthase; CYP71AV1, monooxygenase; DBR2, double-bond reductase 2; ALDH1, aldehyde dehydrogenase; RED1, dihydroartemisinic aldehyde reductase.

AN is produced and store in the GTS, so there is a high correlation between glandular trichome population and AN content in soil-grown *A. annua* plants because AN is synthesized in GTS. The greater the trichome population, the greater is the artemisinin content in the leaves (Kapoor et al., 2007; Arsenault et al., 2010a; Graham et al., 2010). A number of phytohormones have been shown to affect trichome development and/or AN production. Jasmonic acid (JA) induced glandular trichome development while gibberellic acid (GA) induced filamentous trichome development, yet both increase artemisinin production *in planta* (Liu et al., 2009; Maes et al., 2010; Banyai et al., 2011). Other phytohormones such as salicylic acid (SA) and abscisic acid (ABA) also increased artemisinin production, but trichome development was not measured in those studies (Jing et al., 2009; Guo et al., 2009; Pu et al., 2009). On the other hand, 6-benzylaminopurine (BAP) induced more glandular trichome development, but not artemisinin levels in soil-grown plants (Maes et al., 2010).

This study focused on how roots of *A. annua* affect AN metabolite production in the shoots of the plant. In particular, the roles of rooting and the two phytohormones found in shooting medium were measured with respect to glandular trichomes, and artemisinic pathway gene transcription and metabolites.

3.2 Materials and Methods

3.2.1 Maintenance of *In vitro* Cultures

Clonal rooted *Artemisia annua* shoots (clone Sam) were grown in Magenta boxes containing 50 mL semi-solid rooting medium: half-strength Murashige and Skoog basal medium with vitamins (MS, Phytotechnology Laboratories, Prod No. M519), 2% (w/v) sucrose, and 5 g L^{-1} agargellan (Phytotechnology laboratories, Prod. No. A133), at pH 5.8. Unrooted *A. annua* shoots were grown in Magenta boxes containing 50 mL semi-solid shooting medium containing full strength MS salts and vitamins, 0.25 µM α-naphthaleneacetic acid (NAA, Sigma-Aldrich, Cat. No. 0640), 2.5 µM N-6-benzylaminopurine (BAP, Research Organics, Inc., Cat. No. 1106B-1), 3% (w/v) sucrose, and 5 g L^{-1} agargellan at pH 5.8. All cultures were grown at 25°C in a Percival Scientific, Inc. chamber (model AR-66L) equipped with GE Ecolux w/starcoat (F-32T8) and Philips Alto (F32T8) bulbs under continuous light at 70 µmol $m^{-2}s^{-1}$. All cultures were subcultured every two weeks.

3.2.2 Effect of Root Development on Artemisinic Metabolites, Gene Expression, and Trichome Development

Clonal *A. annua* cultures were grown in shooting medium for 2 weeks and then inoculated into either rooting medium or shooting medium (control) in Magenta boxes. Over 16 days, 5 samples from each medium were harvested periodically. When harvesting, each culture was split: one half was used for metabolite analysis and the other half for gene transcript analysis. For trichome analysis, the 2nd fully developed leaf from the shoot apical meristem (SAM) of rooted shoots and a random fully developed leaf from unrooted shoots of 16 day old cultures were used. In shoot cultures there is no obvious SAM, so one cannot use that meristem as a developmental locus.

3.2.3 Effects of NAA and BAP on Level of Artemisinic Metabolites and Trichome Development of Rooted Shoots

Rooted clones were inoculated into 20 x 150 mm Pyrex® glass test tubes containing 10 mL of either rooting medium, rooting medium plus 0.25 µM NAA, rooting medium plus 2.5 µM BAP, rooting medium plus NAA and BAP, or shooting medium that also has the same level of NAA and

BAP, but with full strength MS salts and 3% sucrose. After inoculation, the height of each plantlet was marked on the outside of the test tube indicating where pre-existing growth ends and new growth will begin. Cultures were grown at 25°C under continuous light at 70 µmol $m^{-2}s^{-1}$. After 21 days, cultures were harvested. Each culture was separated according to the mark on the test tube and the fresh weights of the top and bottom shoot of each plant were taken separately along with the fresh weight of the roots. Samples were then extracted and metabolites were analyzed as further described. The experiment was repeated for trichome analysis.

3.2.4 Effects of BAP and NAA on Artemisinic Metabolite Level of Rooted Shoots with Excised Roots

Cultures that had roots for about two weeks were used. These rooted shoots had their roots excised and the shoots were then inoculated into 20 x 150 mm Pyrex® glass test tubes containing 10 mL of either rooting medium or rooting medium plus 2.5 µM BAP or 0.25 µM NAA. After inoculation, the height of each plantlet was marked on the outside of the test tube to demarcate between pre-existing and new growth. After 21 days, cultures were harvested as described previously and extracted as further described.

3.2.5 Metabolite Extraction and Analysis

Cultures were extracted with 1 mL of pentane (HPLC Grade, Fisher Scientific, Cat. No. P399-4) per 0.1 g FW. Test tubes were sealed with Dura Seal stretch film (Diversified Biotech, Cat. No. DS2-500) to decrease evaporation and then sonicated in a chilled water bath (FS60, Fisher Scientific) for 30 minutes. After sonication, extracts were transferred into a clean test tube and dried under a nitrogen stream. Extracts were stored at -20°C until analysis, at which time they were resuspended in pentane and transferred to 1.5 mL vials containing a 100 µL glass insert with octadecane (ARCOS, Cat. No. AC129296050) as an internal standard at a final concentration of 25 µg mL^{-1}. Samples were dried again and then resuspended with 100 µL pentane for AN, AB, and deoxyAN analysis. One microliter of each sample was injected in splitless mode using a HP-5MS column (30 m x 0.25 mm x 0.25 µm) in an Agilent 7890A GC system coupled to an Agilent 5975C MSD with triple-axis detector with the following oven program: injection temperature at 250°C, detection temperature at 280°C, initial temperature at 120°C held for 2 minutes, ramp up to 200°C

at 5°C/min, ramp up again to 300°C at 5°C/min, and then held at 300°C for 5 minutes. Ultra-pure helium carrier gas was used at a flow rate of 1 mL min^{-1}.

For AA and DHAA analysis, samples were transferred to vials as above and resuspended in 20 µL of pyridine (CHromasolv® Plus, Sigma-Aldrich, Cat. No. 270407) and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Restek, Bellefonte, PA; Cat. No. 35605) solution (1:1 v/v) and 50 µL pentane (Zhang et al., 2010). Samples were analyzed in the same GC/MS as described above with the following oven program in splitless mode: injection temperature at 250°C, detection temperature at 280°C, initiation temperature at 125°C, and ramp up to 300°C at 5°C/min (Zhang et al., 2010). Ultra-pure helium carrier gas was used at a flow rate of 1 mL min^{-1}.

Metabolites were identified via retention time and signature ion peak mass spectra as compared to the external standards (AN, Sigma Chemical, St. Louis, MO; AB, gift of Dr. Nancy Acton, Walter Reed Army Research Institute, Silver Spring, MD; AA, Apin/chemical, Abingdon, UK) and mass spectra presented in Zhang et al. (2010). DHAA was identified using the mass spectra published by Zhang et al. (2010) from synthesized DHAA, but it is labile under long term storage so DHAA is quantitated based on AA standards and expressed as AA equivalents (Arsenault et al., 2010). DeoxyAN was identified using the mass spectra from the NIST GC/MS library (2008), but since no deoxyAN could be obtained, it was quantitated based on the AN standard.

3.2.6 Trichome Analysis

To compare trichome development of 16 day old rooted shoots and unrooted shoots, the 2nd fully formed leaf from rooted shoots and a random leaf from unrooted shoots were taken for analysis. To compare trichome development of new and old growth, the 2nd and 9th fully formed leaves from the apical meristem were chosen for trichome analysis of new (2nd leaf) and old (9th leaf) growth. Each leaf was placed on a glass slide with 3 drops of Type F immersion liquid (Leica Microsystems CMs GmbH, Cat. Nr. 11513 859) to prevent drying of the leaf. Four drops of silicon vacuum grease were placed at the corners below the cover slip to ensure that the leaf sample was level. After placing the cover slip, nail polish (Colorstay Revlon 2 Always On Sealant) was applied around the cover slip to keep the sample in place and to prevent evaporation. Using a confocal microscope (Leica TCS SP5: Broadband Confocal, Leica Microsystems) three pictures of

each leaf sample were taken and then stacked. Pictures were exported to ImageJ (http://rsbweb.nih.gov/ij/) for trichome population and sac size analysis. For trichome population analysis, an average number of trichomes per mm^2 of the three pictures was taken per leaf sample. For trichome sac size analysis, the area of six randomly chosen trichomes was taken for each picture. Then an average sac size of the three pictures was measured per leaf sample. There were at least 5 leaf samples per condition. Percent leaf area occupied by trichome was also calculated:

% trichome covered leaf area = [(# of trichomes x average trichome size (mm^2)) / total leaf image area (mm^2)] [100].

3.2.7 Gene Transcript Analysis (qPCR)

Immediately after harvesting, plant samples were frozen with liquid nitrogen and stored at -80°C. To extract RNA, plant samples were ground in a Cryo Homogenizing System (Fisher Scientific, Cat. No. 02 515 500) and RNA extracted with Plant RNA Purification Reagent (Invitrogen, Cat No. 12322-012) and purified with Turbo DNA-*free* kit (Ambio, Cat No. AM1907). The purified RNAs were reverse-transcribed into cDNA using the DyNAmo™ cDNA synthesis kit (Thermo Scientific, F-470L). Transcripts were analyzed via qPCR using the iQ™ SYBR®Green Supermix (Bio-rad, Cat. No. 170-8882) and the Bio-rad iCycle (Bio-Rad, Hercules CA) under a 3-step amplification program. For each cycle, there is a denaturation step at 94°C, annealing step at 53°C, and extension step at 72°C. There was a total of 35 cycles followed by a melting-curve analysis. Primers used for analysis are presented in Table 3.1 with 18S used as internal reference. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ comparative method (Cikos et al., 2007) and presented as fold change relative to day 0.

Table 3.1: Primer sequences for qPCR of *A. annua* shoots.

Gene	Direction	Sequence (5' → 3')	Base Pairs	Product Length
ADS	Forward	ATACAACGGGCACTAAAGCAACC	23	297 bp
ADS	Reverse	GAAAACTCTAGCCCGGGAATACTG	24	297 bp
CYP	Forward	GGGGTTAGGGATTTAGCCAGAA	22	218 bp
CYP	Reverse	AATTGCCTCCAGTACTCACCATAA	24	218 bp
DBR2	Forward	GCTTTCTTATTACCGAGGGGACTA	24	228 bp
DBR2	Reverse	AAATGGGCTTGCTTGTTGATGATA	24	228 bp
18S	Forward	TCCGCCGGCACCTTATGAGAAATC	24	219 bp
18S	Reverse	CTAAGAACGGCCATGCACCACCAC	24	219 bp

3.2.8 Statistical Methods

All experiments had at least five replicates. Metabolite and trichome data were averaged and analyzed using Student's t-test. Real-time PCR data was analyzed using Mann-Whitney U test (Yuan et al., 2006).

3.3 Results

Prior reports showed that when *Artemisia annua* shoots were grown in rooting medium, they produced more artemisinin (AN) than shoots grown in shooting medium. Thus, the dynamics of artemisinic metabolite production and transcript levels in rooted and unrooted shoots were measured first. Subsequently these same shoot characteristics were measured in plants grown *in vitro* with variations in hormones and nutrient levels. Comparisons were also made between mature and immature shoot tissues.

3.3.1 Effects of Rooting on Artemisinic Metabolites, Transcription of Genes in the Artemisinin Biosynthetic Pathway, and Glandular Trichome Development

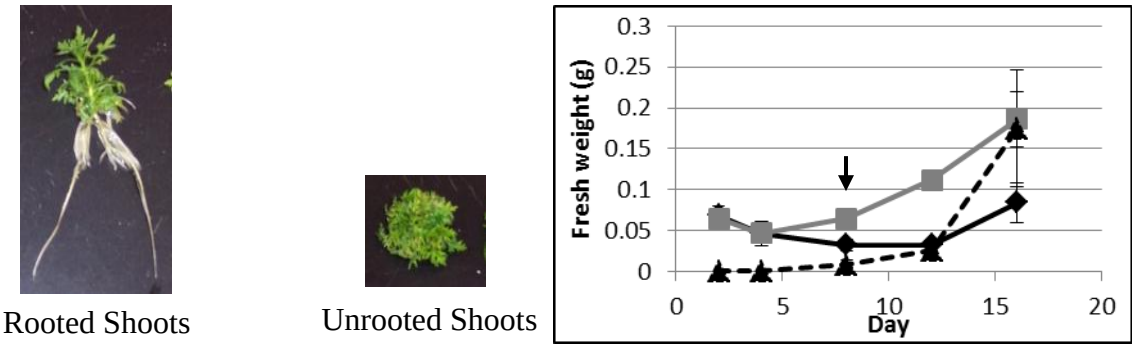


Figure 3.2: *A. annua* shoots grown in either rooting or shooting medium for 16 days. Grey solid line, unrooted shoots; black solid line, shoots of rooted shoots; black dotted line, roots of rooted shoots. Arrow indicates appearance of roots. n = 5.

When unrooted shoots (shoots that were maintained in shooting medium) were inoculated into either rooting medium or shooting medium (control) and then grown for 16 days, roots first appeared after about 8 days (Fig. 3.2, arrow). After 16 days, the cultures grown in rooting medium developed long roots and shoots began to elongate (Fig. 3.2). In contrast, cultures grown in shooting medium did not develop any roots and continued to grow laterally, producing shrubby

plantlets (Fig. 3.2). Biomass growth yields were also quite different for the two types of cultures. The shoot mass of unrooted shoots (Fig. 3.2, grey line) increased reaching 0.18 g FW on day 16, while shoot mass of rooted shoots (Fig. 3.2, black solid line) barely increased by day 16 to 0.08 g FW. On the other hand, once roots emerged on day 8, the root mass of rooted shoots increased significantly by day 16 to 0.17 g FW. By day 16, total biomass of rooted shoots was about 50% greater than total biomass of unrooted shoots (Fig. 3.2).

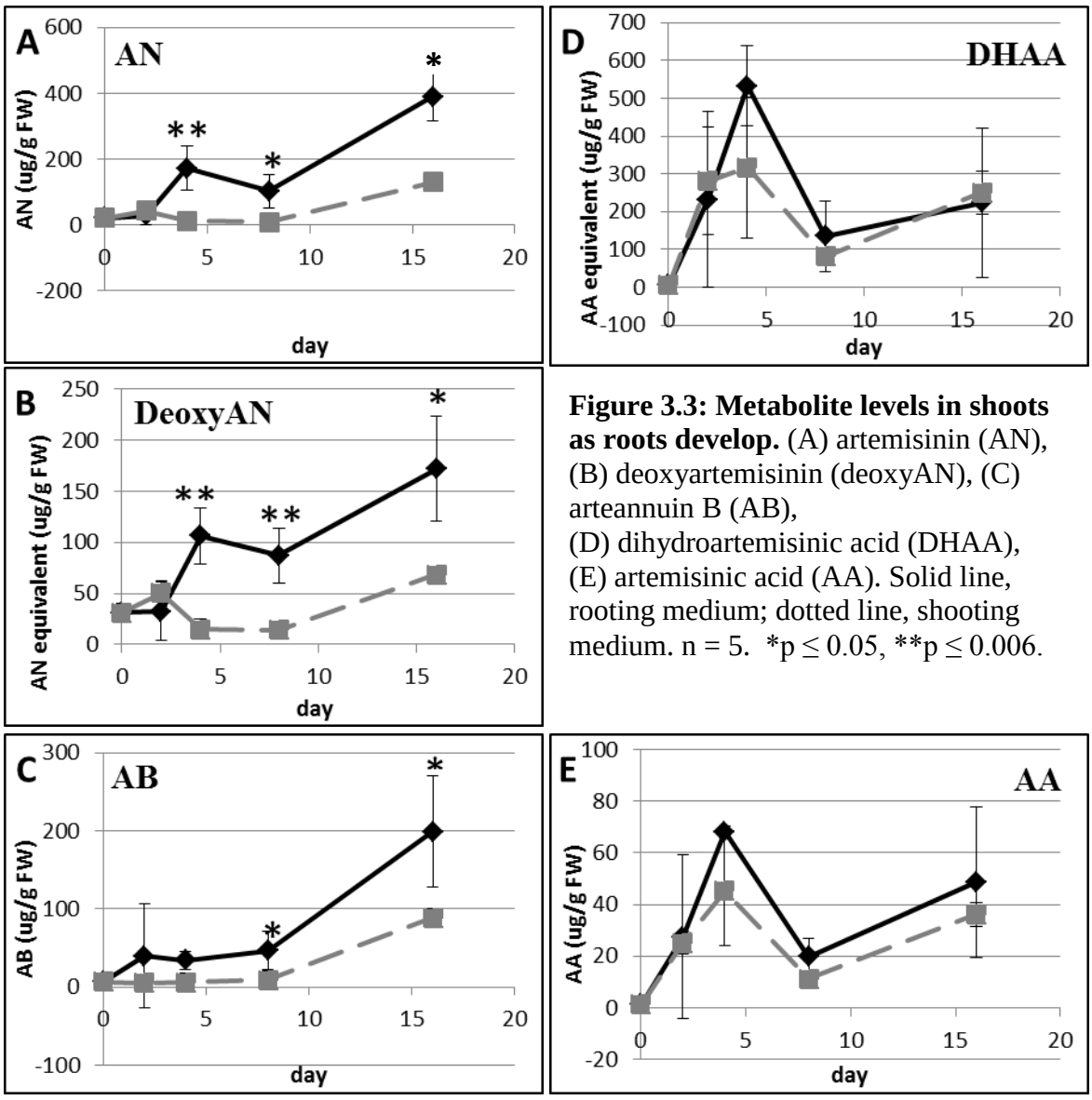


Figure 3.3: Metabolite levels in shoots as roots develop. (A) artemisinin (AN), (B) deoxyartemisinin (deoxyAN), (C) arteannuin B (AB), (D) dihydroartemisinic acid (DHAA), (E) artemisinic acid (AA). Solid line, rooting medium; dotted line, shooting medium. n = 5. *$p \leq 0.05$, **$p \leq 0.006$.

After 4 days, both AN and deoxyAN were significantly greater in shoots of rooted cultures than unrooted shoots (Fig. 3.3A, B). By day 8 when roots were first visible, rooted shoots had significantly greater amounts of all three end products, than unrooted shoots (Fig. 3.3 A-C). In

contrast, neither of the measured precursors, AA and DHAA, was significantly different. When the artemisinic metabolites were measured at 16 days, significantly more AN, deoxyAN, and AB were found in rooted shoots (390.3, 171.7, and 199.1 µg g FW^{-1}, respectively) than in unrooted shoots (131.3, 68.4, and 88.6 µg g FW^{-1}, respectively), but DHAA and AA levels in both cultures stayed about the same (Fig. 3.3). Compared to unrooted shoots, the shoots of rooted cultures accumulated significantly more end products (AN, deoxyAN, AB), than precursors (DHAA, AA) of the AN biosynthetic pathway (Fig. 3.3).

Next, transcript levels of three genes in the pathway were measured. ADS and CYP71AV1 (CYP) are the first two enzymes in the AN biosynthetic pathway, so it was important to determine how rooting affects transcript levels of these genes. DBR2 was also measured because it functions only in the branch that leads to AN and deoxyAN production. Any differences detected in *dbr2* transcripts will help determine if there is a shift to either the AA-AB branch or the DHAA-AN-deoxyAN branch of the pathway in response to rooting. As in the previous experiment, unrooted shoots were inoculated into either rooting medium or shooting medium (control). Transcripts of *ADS, CYP,* and *DBR2* were measured using qPCR on days 2, 4, 8, 12, and 16 after inoculation. Although *ads* transcripts on day 4, 8, 12, and 16 increased relative to day 0, the difference in rooted shoots compared to unrooted shoots was not statistically different (Fig. 3.4A). Similar to the response for *ADS,* transcripts of *CYP* and *DBR2* also showed no significant differences between rooted and unrooted shoots (Fig. 3.4B, C). These results suggested that rooting media does not affect the transcription of these genes, and that the shift in AN level must be due to something other than transcriptional changes in these particular genes.

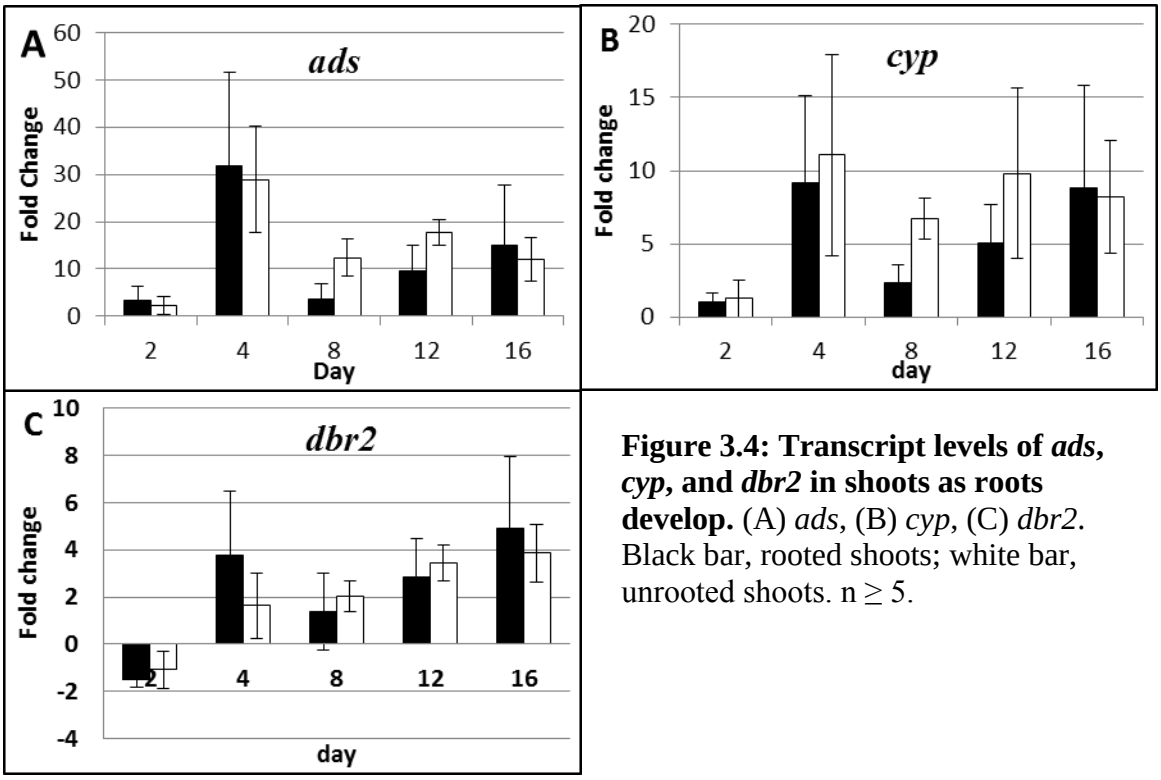


Figure 3.4: Transcript levels of *ads*, *cyp*, and *dbr2* in shoots as roots develop. (A) *ads*, (B) *cyp*, (C) *dbr2*. Black bar, rooted shoots; white bar, unrooted shoots. $n \geq 5$.

AN is produced and stored in the glandular trichomes. Since rooting did not seem to affect transcript levels of the AN pathway genes, it may instead be affecting trichome development. Using confocal microscopy, the number and size of trichomes were measured on 16 day old *in vitro* cultured shoots and rooted shoots. There was no significant difference in trichome populations on leaves of rooted and unrooted shoots (29.9 trichome/mm^2 and 30.9 trichome/mm^2, respectively; Fig. 3.5A). On the other hand, glandular trichomes of rooted shoots were significantly larger than those of unrooted shoots (2,080 μm^2 and 1,317 μm^2, respectively; Fig. 3.5B). It appeared that roots were therefore affecting the size, but not necessarily the number, of trichomes on leaves of *A. annua*.

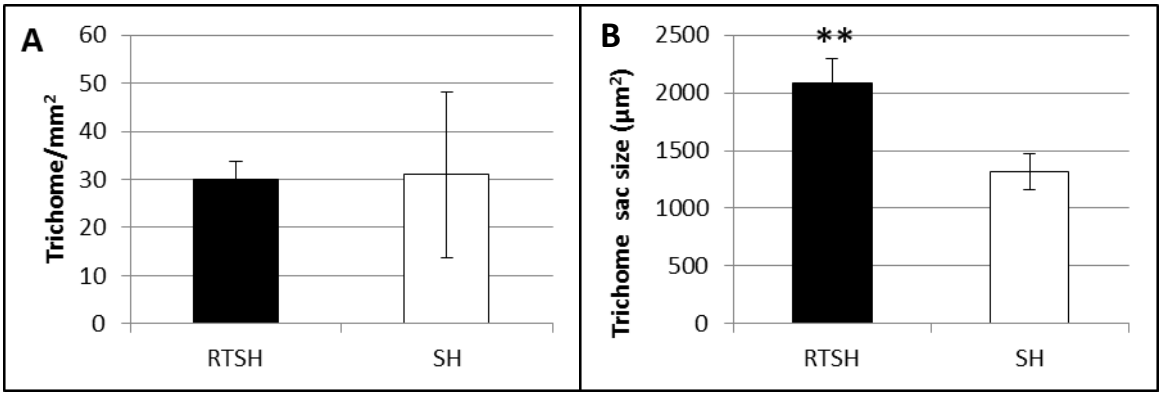


Figure 3.5: Trichome density and sac size of 16 day old rooted shoots and unrooted shoots. (A) trichome population density, (B) trichome sac size. RTSH = rooted shoots; SH = unrooted shoots. n = 5. **$p \leq 0.001$.

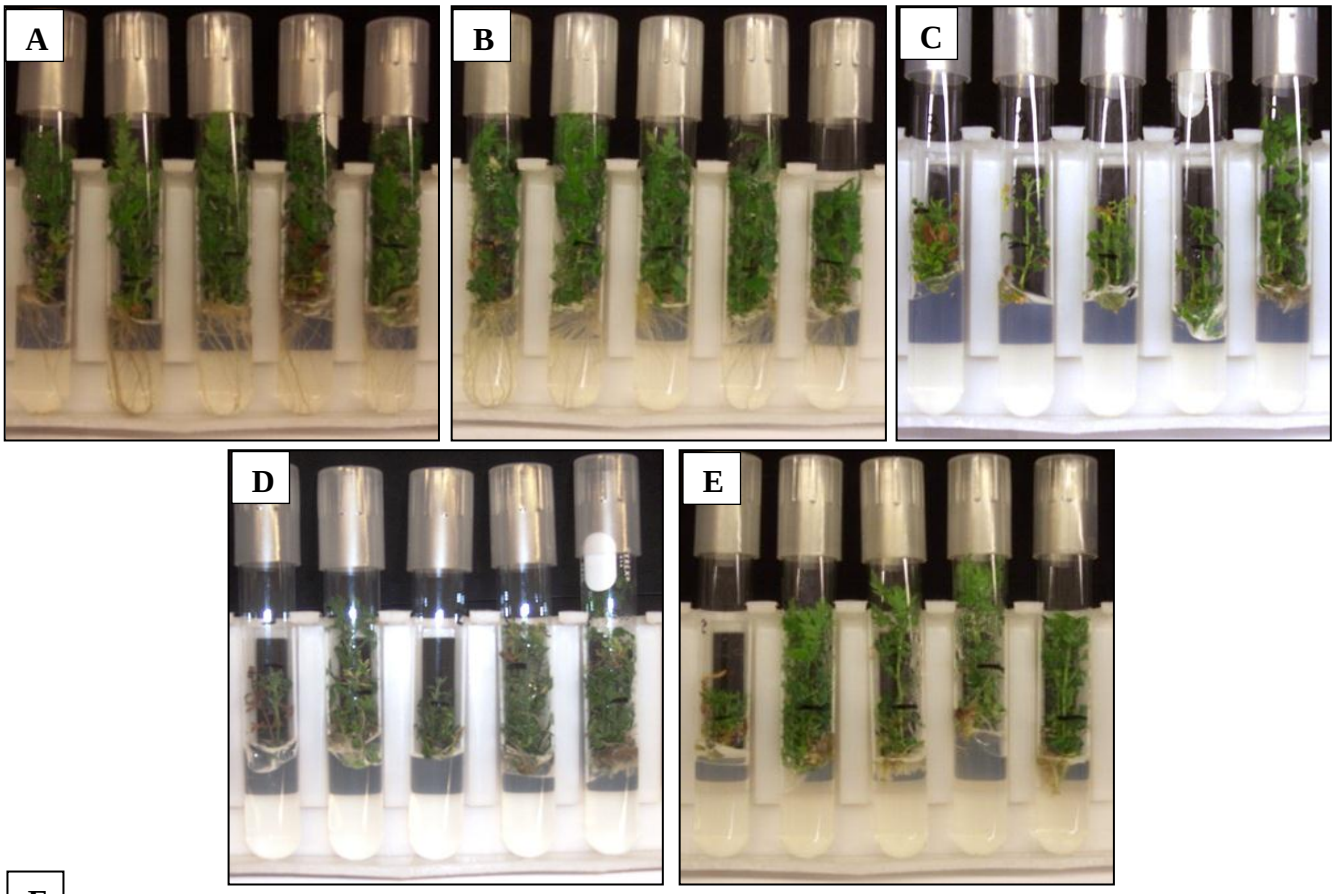
3.3.2 Effects of NAA and BAP on Artemisinic Metabolite and Trichome Development of Rooted Shoots

Although roots might play a role in the artemisinin biosynthetic pathway, it is also possible that the phytohormones in the shooting medium, BAP and NAA, are inhibiting the pathway and causing lower levels of AN, deoxyAN, and AB in unrooted shoots. To determine if either roots and/or hormones are important in artemisinic metabolite production, two week old rooted shoots were inoculated into rooting medium ± NAA, ± BAP, or ± both hormones (Fig. 3.6A-D). Another difference between rooting and shooting medium is that rooting medium only contains ½ MS salts and vitamins plus 2% sucrose while shooting medium contains full strength MS salts and vitamins plus 3% sucrose. To separate the hormonal responses from responses to other medium constituents, two week old rooted shoots were inoculated into shooting medium and compared to the rooted shoots inoculated into rooting medium that contained both NAA and BAP (Figure 3.6D, E). Plantlets were grown for three weeks and then growth, artemisinic metabolites, and trichome development were measured and compared in the new and old growth. It was assumed that if there was an effect from either hormone or other medium constituents (MS salts and sucrose concentration) then it would occur in the new growth, which developed after inoculation into the various media formulations.

After three weeks, rooted shoots in rooting medium and rooting medium plus NAA showed similar growth morphology (Fig. 3.6A, B). There were many leaves, lengthened internodes, and roots extending to the bottom of the test tubes. On the other hand, rooted shoots grown in rooting

medium + BAP (Fig. 3.6C), rooting medium plus both hormones (Fig. 3.6D), and shooting medium (Fig. 3.6E) did not grow as tall as those grown in either rooting medium (Fig. 3.6A) or rooting medium + NAA (Fig. 3.6B). The roots in the latter media (Fig. 3.6C, D, E) were also thick and short; however, the fresh mass of the roots was unchanged from those grown in rooting medium or in rooting medium with NAA (Fig. 3.6F). Although different between cultures, shoot biomass correlated well with the height of the plants shown in Fig. 3.6A-E (Fig. 3.6F).

Artemisinic end products, AN, deoxyAN, and AB, were also measured in both new and old shoot growth, and there was significantly less AN in the new growth than in the old growth of rooted shoots grown in rooting medium (Fig. 3.7A). There was also less AN in the new growth of cultures grown in NAA and with both hormones. Similarly, there was less AB in new growth than in old growth (Fig. 3.7C). There was less deoxyAN in the new growth than in the old growth of rooted shoots in rooting medium and rooting medium plus NAA (Fig. 3.7B), but there was either similar or more deoxyAN in the new growth than old growth in rooted shoots grown in medium containing BAP (Fig. 3.7B). On the other hand, there were more of the precursors, DHAA and AA, in the new growth compared to the old growth in all the cultures (Fig. 3.7D, E). Interestingly, AA was barely detected in the old growth of cultures grown in rooting medium and rooting medium plus NAA. These data show that in the newly grown shoot precursors were made, but not yet converted into their respective end products. On the other hand, in the older portion of the shoot, precursor levels were relatively low, suggesting they were already converted into end products; there were more end products and fewer precursors in the old tissue and vice versa in the new tissue.



F

Medium	Roots (g)	Root Morphology	Old Growth (g)	New Growth (g)	Total Shoot Growth (g)
RTM	0.15 ± 0.03	gravitropic, long, thin	0.19 ± 0.06	0.18 ± 0.03	0.35 ± 0.08
NAA	0.18 ± 0.05		0.18 ± 0.05	0.24 ± 0.06	0.42 ± 0.08
BAP	0.17 ± 0.04	agravitropic, short, thick	0.13 ± 0.07	0.11 ± 0.06	0.26 ± 0.10
Both	0.17 ± 0.06		0.12 ± 0.06	0.09 ± 0.06	0.23 ± 0.08
SHM	0.16 ± 0.04		0.15 ± 0.05	0.16 ± 0.08	0.29 ± 0.11

Figure 3.6: Growth of rooted shoots in rooting medium containing NAA and/or BAP after three weeks. (A) rooting medium; (B) rooting medium plus NAA; (C) rooting medium plus BAP; (D) rooting medium plus NAA and BAP; (E) shooting medium. n = 14. Abbreviations: RTM, rooting medium; NAA, rooting medium plus 0.25 µM NAA; BAP, rooting medium plus 2.5 µM BAP; both, rooting medium plus 0.25 µM NAA and 2.5 µM BAP; SHM, shooting medium.

It was assumed that any effect of the phytohormones or other medium constituents would be visible in only the new growth, but that was apparently not the case. When compared to the old growth of cultures grown in rooting medium, there was significantly more AN in the old growth of cultures grown in rooting medium plus NAA and in medium containing both hormones (Fig. 3.8A). Interestingly, the level of AN was similar in the new growth as well as total growth of all

the cultures (Fig. 3.8A). In contrast to AN, there was no difference in the amount of deoxyAN in the old growth, but there was more deoxyAN in the new growth and total growth of cultures grown in the presence of BAP (Fig. 3.8B). Although there was more AB in the new growth of cultures grown in the presence of either of the hormones, there was less AB in the old growth (Fig. 3.8C). Overall, cultures grown in the presence of NAA and/or BAP had significantly less AB in the old and total growth. In the presence of BAP, there was more DHAA and AA in the old, new, and total growth (Fig. 3.8D, E), but the effect of NAA varied. There was slightly more DHAA in the total growth of cultures grown in rooting medium plus NAA compared to cultures grown in just rooting medium (Fig. 3.8D). In contrast, rooted shoots grown in rooting medium plus NAA had significantly less AA in the new growth as well as in the total growth (Fig. 3.8E). These data suggest that, in the DHAA-AN-deoxyAN branch, NAA might play a role in the DHAA-AN sub-branch, while BAP affects the DHAA-deoxyAN sub-branch. For the AA-AB branch, the effect of each phytohormone was different. BAP seems to be inhibiting the conversion of AA to AB so although there was a high level of AA, the level of AB was significantly less compared to cultures grown in just rooting medium. On the other hand, NAA seemed to be inhibiting the production of AA, the precursor to AB, resulting in a low level of AB.

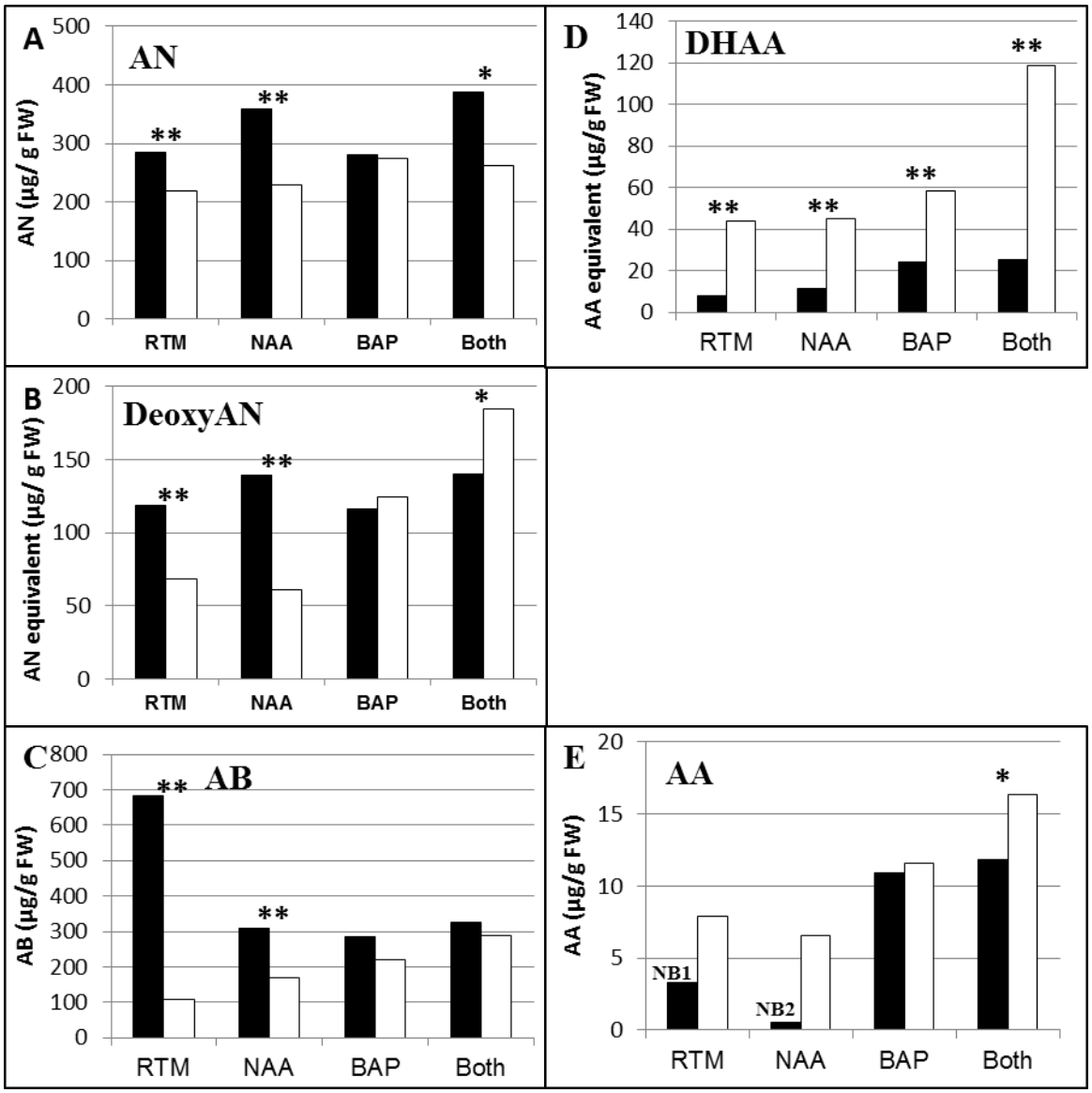


Figure 3.7: Comparison of metabolite levels between new and old growth shoots of rooted shoots in rooting medium containing NAA and/or BAP. (A) AN, (B) deoxyAN, (C) AB, (D) DHAA, (E) AA. Black, old growth; white, new growth. n = 14. For comparison between new and old growth: *$p \leq 0.05$;**$p \leq 0.009$. NB1 indicates that only 1 out of 14 plantlets has artemisinic acid in the old growth; NB2 is actually out of 14 plantlets none has artemisinic acid in the old growth. Abbreviations: RTM, rooting medium; NAA, rooting medium plus 0.25 µM NAA; BAP, rooting medium plus 2.5 µM BAP; Both, rooting medium plus 0.25 µM NAA and 2.5 µM BAP.

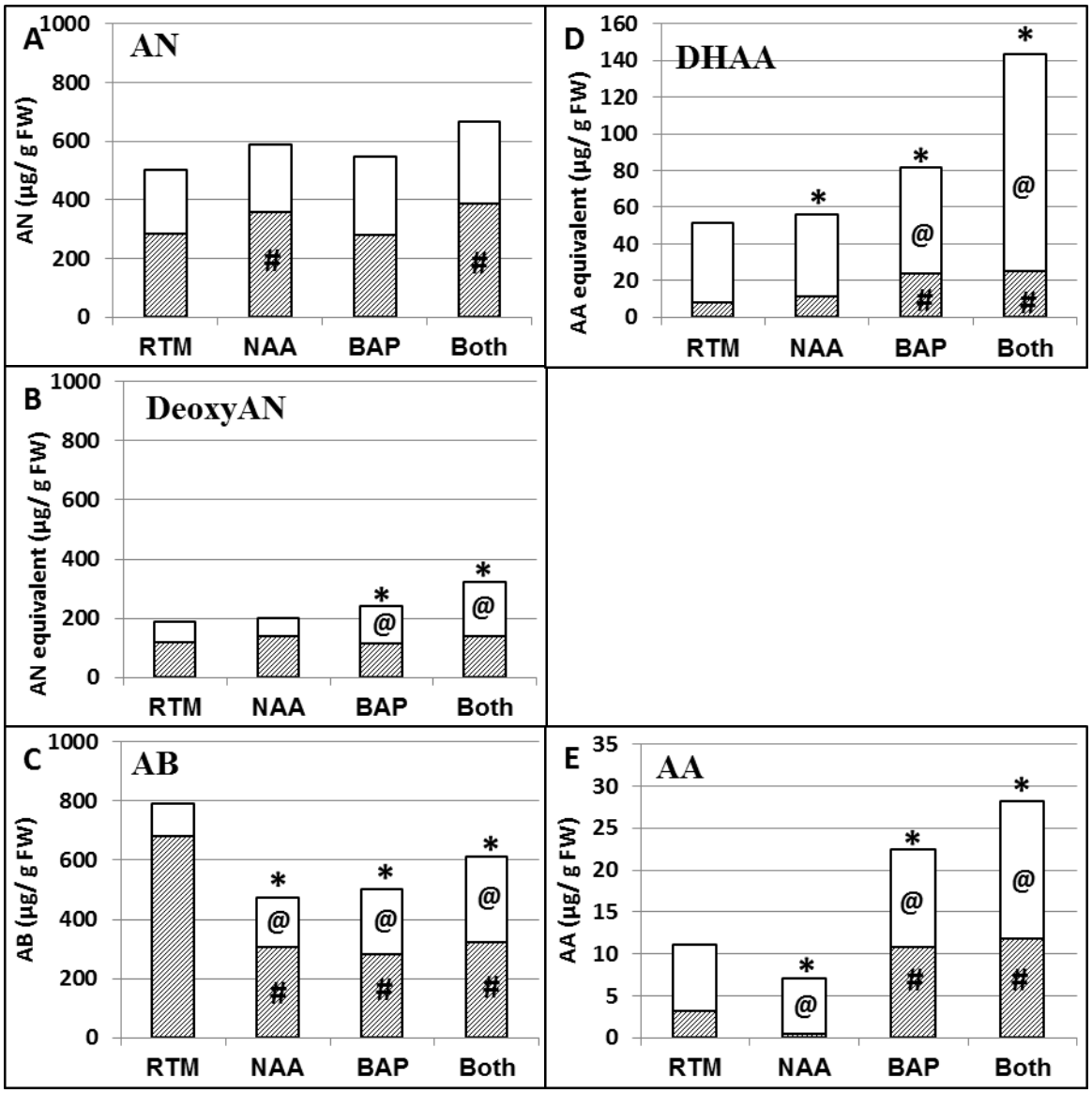


Figure 3.8: Metabolite levels of rooted shoots in rooting medium containing NAA and/or BAP. (A) AN, (B) deoxyAN, (C) AB, (D) DHAA, (E) AA. Shaded, old growth; white, new growth. n = 14. For comparison to old growth of rooting medium: # $p \leq 0.05$; for comparison to new growth of rooting medium: @ $p \leq 0.05$; for comparison to entire plant of rooting medium: *$p \leq 0.05$. Abbreviations: RTM, rooting medium; NAA, rooting medium plus 0.25 µM NAA; BAP, rooting medium plus 2.5 µM BAP; Both, rooting medium plus 0.25 µM NAA and 2.5 µM BAP.

To determine if the other media constituents (MS salts, vitamins, and sucrose) had any effect on artemisinic metabolite levels of rooted shoots, it was necessary to compare cultures grown in rooting medium containing both the hormones with cultures grown in shooting medium. Similar to cultures grown in the other type of medium, there was either more or a similar level of end

products (AN, deoxyAN, and AB) in the old growth compared to the new growth (Table 3.2). Conversely, there was either less or a similar amount of precursors (DHAA and AA) in the old growth compared to the new growth. This further suggested that in the new growth the precursors are not yet converted to their respective end products, but in the old growth most of the precursors are already converted to end products. While the higher concentration of MS salts, vitamins, and sucrose in the shooting medium seemed to stimulate the production of only AB in the old growth (Table 3.2), it did not affect the level of the other metabolites in either the old or the new growth.

Table 3.2: Metabolite levels in old and new growth of rooted shoots after growth in rooting medium with NAA + BAP or in shooting medium.

	AN (µg/g FW)		DeoxyAN (µg/g FW)		AB (µg/g FW)		DHAA (µg/g FW)		AA (µg/g FW)	
Medium	**Old**	**New**	**Old**	**New**	**Old**	**New**	**Old**	**New**	**Old**	**New**
Both	387a,x	263b,x	140a,x	185b,x	324a,x	289a,x	25a,x	118b,x	12a,x	16b,x
SHM	417a,x	263b,x	154a,x	144a,x	533a,y	314b,x	31a,x	79b,x	13a,x	14a,x

Abbreviations: Both, rooting medium plus 0.25 µM NAA + 2.5 µM BAP; SHM, shooting medium; Old, shoot growth prior to inoculation; New, shoot growth post inoculation. Letters indicate statistical differences at $p \leq 0.05$, n=14; for comparison between old and new growth of cultures grown in the same medium: a,b; for comparison between old growth of cultures grown in either rooting medium plus NAA + BAP (both) or shooting medium: x,y; for comparisons between new growth of cultures grown in either rooting medium plus NAA + BAP (both) or shooting medium: x,y.

3.3.3 Effects of NAA, BAP, and Other Media Constituents on Glandular Trichome Development

Since AN and the other artemisinic metabolites are stored in the glandular trichomes and it appears that hormones are playing a role in the AN biosynthetic pathway, it is possible that glandular trichome development also may be affected. As in the previous experiment, two week old rooted shoots were inoculated into the five test media and after three weeks, two fully expanded leaves, were analyzed. The second fully formed leaf from the shoot apical meristem was taken as representative of new growth and the ninth fully formed leaf, which was below the inoculation line, was taken as representative of old growth. Each set of leaves was viewed under a confocal microscope and pictures were taken and then stacked; glandular and filamentous trichomes are shown in Fig. 3.9A.

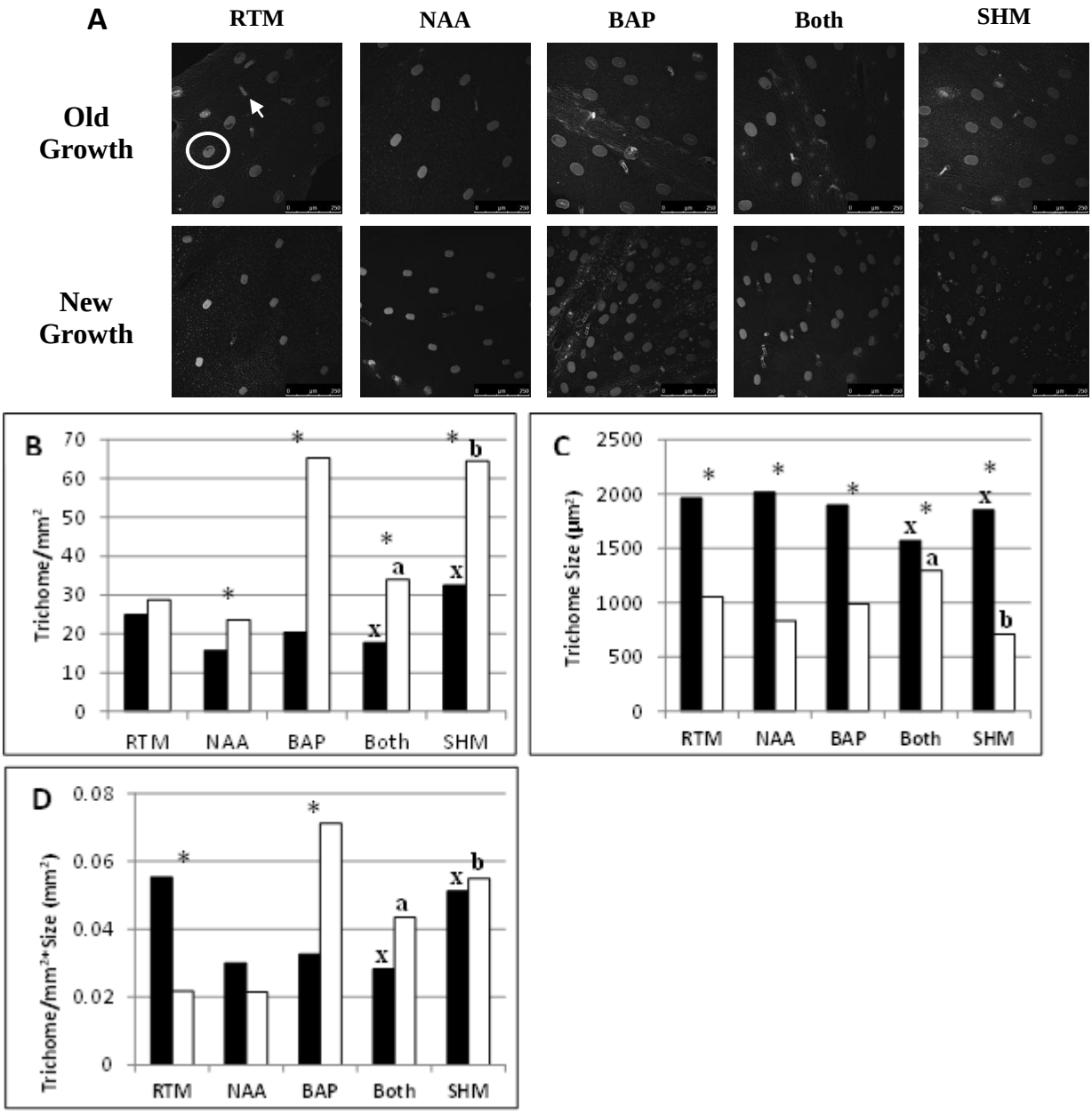


Figure 3.9: Glandular trichome (GT) development of rooted shoots in rooting medium containing NAA and/or BAP, or in shooting medium. (A) trichomes under a confocal microscope; RTM, old growth: white circle, glandular trichome; white arrow, filamentous trichome. (B) GT population density, (C) GT trichome sac size, (D) percent leaf surface occupied by GTss. Black, old growth; white, new growth. For comparison between new and old growth: n= at least 5 rooted shoots and *$p \leq 0.05$. For comparison between new growth of medium containing both hormones (Both) and shooting medium (SHM), a,b. For comparison between old growth of medium containing both hormones (Both) and shooting medium (SHM), x,y. Abbreviations: RTM, rooting medium; NAA, rooting medium plus 0.25 µM NAA; BAP, rooting medium plus 2.5 µM BAP; Both, rooting medium plus 0.25 µM NAA and 2.5 µM BAP; SHM, shooting medium.

When rooted shoots were transferred to rooting medium, there was no significant change in trichome number in the new growth, but trichome size was smaller (Fig. 3.9B, C). When rooted cultures were transferred to rooting medium containing NAA, BAP or Both, trichome numbers significantly increased in the new growth compared to the old growth; these increases also occurred even when transfer was to shooting medium (Fig. 3.9B). Although there were more trichomes in new growth in plants transferred to nearly all conditions, trichomes in all cultures were significantly smaller than in the older leaves (Fig. 3.9C). For example, the average trichome sac size of the new growth was 1,056 μm^2 compared to 1,966 μm^2 for the trichome sac size of the old growth in rooting medium.

The total area of a leaf occupied by trichomes was also calculated to measure the overall impact of each hormone on trichome development (Fig. 3.9D). In rooted shoots grown in rooting medium, the percent leaf area occupied by trichomes was significantly greater in the old growth than in the new growth, 4.8% and 2.8%, respectively, but in the presence of BAP the results were reversed. Area occupied by trichomes was greater in the new growth than in the old growth of BAP-grown cultures, 8.1% and 3.8%, respectively. Although shooting medium and rooting medium plus both hormones contained BAP, the percent leaf area occupied by trichomes was smaller in cultures grown in these media than in cultures grown in rooting medium containing only BAP, suggesting that NAA reduced the effect of BAP.

Other media constituents also affected trichome development (Fig. 3.9B-D). In shooting medium, where there were greater amounts of salts, vitamins, and sucrose, there were more glandular trichomes (Fig. 3.9B), but they were significantly smaller in the new growth (Fig. 3.9C). Overall, however, this increase in media constituents in SHM (vs. Both) resulted in a greater percentage of leaf surface area occupied by trichomes (Fig. 3.9 D). In contrast, there were no differences in glandular trichome population density, sac size, or percent leaf surface area occupied by trichomes in the old growth. This suggested that the higher concentration of media constituents did not affect trichome development since these trichomes were already formed before inoculation,

3.3.4 Effects of BAP and NAA on Rooted Shoots with Their Roots Removed

It appeared that both BAP and NAA affected the production of artemisinic metabolites, so it was important to determine whether the roots or hormones were more important in regulating the AN

pathway. By using rooted shoots that had their roots removed, it was posited that if hormones had a greater influence than roots, then in the presence of hormones the rootless shoots should yield greater artemisinic compounds than rooted shoots. If BAP, and not the roots, plays a critical role in the AN pathway, then the metabolite levels should be similar to those found in rooted shoots with their roots intact grown with BAP (Fig. 3.10A, C). Similarly, if NAA plays a role in the AN pathway, then there should be a similar amount of AN and deoxyAN and less AB in cultures grown with NAA than in those grown in NAA-free medium (Fig. 3.10A, C).

Even though the biomass of cultures grown in rooting medium and rooting medium plus BAP was similar after three weeks, the cultures grown in rooting medium developed roots and elongated, but cultures grown in rooting medium plus BAP did not develop any roots, and instead of elongating grew bushy side shoots (Table 3.3). There was significantly more AN in cultures grown in rooting medium versus rooting medium plus BAP, 449 μg/ g FW and 179 μg/ g FW, respectively (Fig. 3.10B), but there was no difference in either deoxyAN or AB, the other measured end products, or in the precursors, AA and DHAA. When all the end products and/or precursors were summed (Fig. 3.10D), it showed that, although the amount of precursors in both cultures was similar, there were more end products in cultures grown in rooting medium without BAP. These results suggested that the presence of roots, not BAP, enhanced the conversion of precursors to end products, and that this conversion was inhibited once roots were removed.

On the other hand when shoots with roots excised were inoculated into rooting media ± NAA, roots formed on all shoots and there was no significant difference in any of the 5 measured metabolites. Although shoot biomass did not differ, there was significantly more root biomass in cultures with NAA (Table 3.3). Although there was about three times the amount of AN compared to DHAA, the amount of AN was 5.6 times greater than deoxyAN. Totally the end products of that pathway branch were 3-4 times greater than their precursor DHAA. In contrast neither AA nor AB were detectable in shoots from cultures grown in either medium. Although the cultures with NAA had more than four times the root biomass of the NAA-free cultures, there was no significant difference in artemisinic metabolites. On the other hand, NAA fed cultures had significantly more AN than cultures fed BAP.

Table 3.3: Shoot and root growth of rooted shoots with roots removed in rooting medium containing BAP or NAA.

Medium	Shoot (g)	Root (g)
RTM[a]	0.26 ± 0.05	0.16 ± 0.04
RTM + 2.5 µM BAP	0.29 ± 0.11	N/A
RTM[a]	0.213±0.054	0.105±0.048
RTM + 0.25 µM NAA	0.213±0.054	0.897±0.021

[a] BAP and NAA experiments were run at different times, so each had its own separate RTM control.

Abbreviations: RTM, rooting medium; N/A, not applicable because no roots formed. n = at least 5 plants.

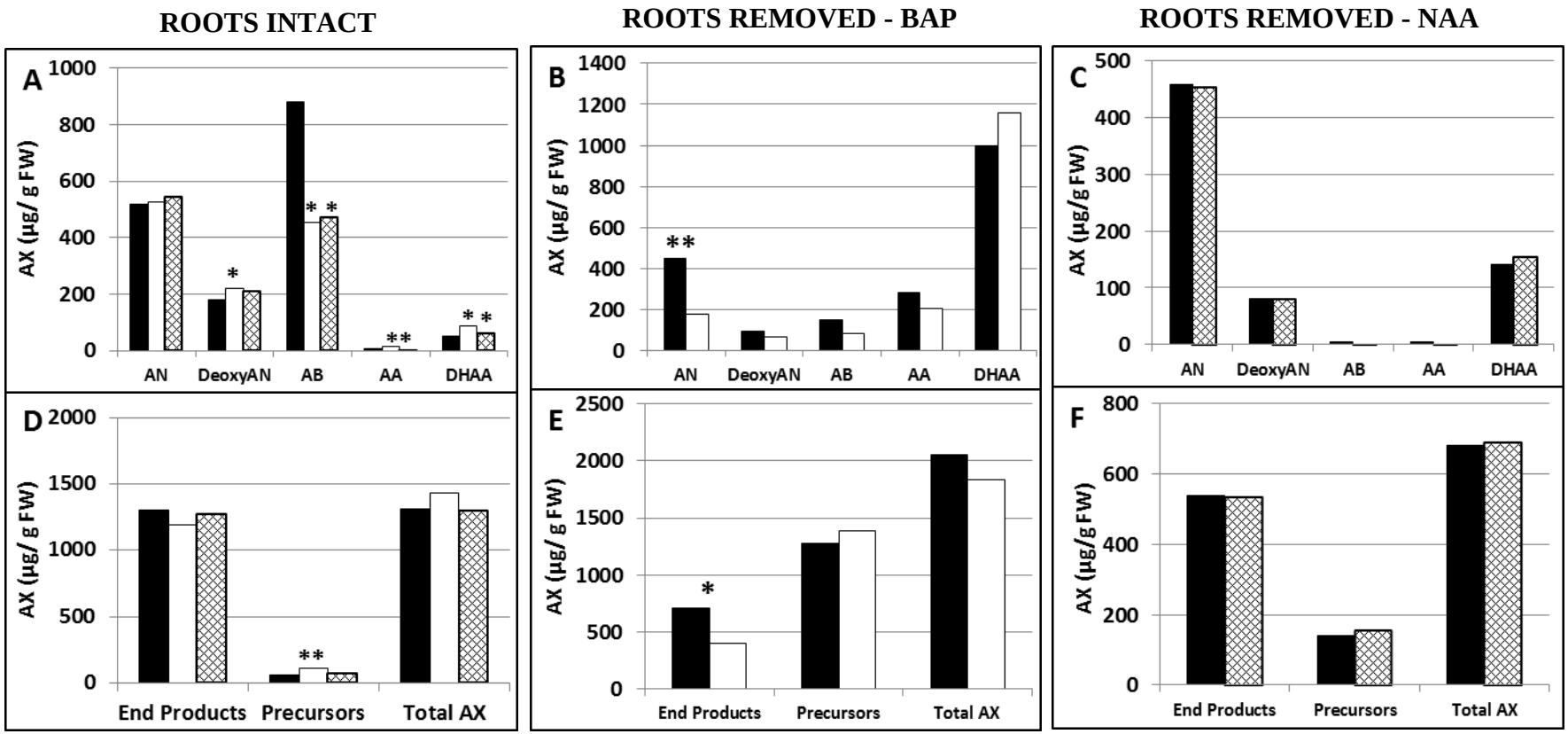


Figure 3.10: Metabolite levels of rooted shoots with roots intact or removed in rooting medium containing BAP or NAA. (A) individual metabolites with roots intact, (B) individual metabolites with roots removed grown in medium containing BAP, (C) individual metabolites with roots removed grown in medium containing NAA, (D) total metabolites with roots intact, (E) total metabolites with roots removed grown in medium containing BAP, (F) total metabolites with roots removed grown in medium containing NAA. *$p \leq 0.05$. $n \geq 5$. Black, rooting medium; white, rooting medium plus 2.5 µM BAP; hatched, rooting medium plus 0.25 µM NAA. Abbreviations: AN, artemisinin; deoxyAN, deoxyartemisinin; AB, arteannuin B; AA, artemisinic acid; DHAA, dihydroartemisinic acid; AX, all artemisinic metabolites.

3.4 Discussion

As leaves emerge, expand, and fully develop, trichomes form and enlarge and much of the data shown in this study correlates with that developmental process. Trichomes in the young immature leaves near the top of the plants were smaller, but in rooted shoots there were more of them compared to the older mature leaves nearer the bottom of the plants, which had fewer, but larger, trichomes. This was not surprising since trichomes in the new growth were newly developed and therefore need time to expand. The trichomes in the old growth had time to expand and accumulate secondary metabolites, and therefore were much larger.

Lommen et al., (2006) previously noted that levels of the AN precursor, DHAA levels were highest in newly formed leaves, declining as the leaves matured. As DHAA declined, there was an increase in AN with the highest level measured in senesced (brown) leaves (Lommen et al., 2006). We observed a similar pattern in metabolite levels in *A. annua in vitro* rooted shoots. There were more precursors, DHAA and AA, but fewer end products, AN, deoxyAN, and AB, in the young leaves than in the mature leaves. Young leaves need time to convert precursors to end products, so it makes sense that there are more precursors and fewer end products. Conversely, in the mature leaves, most of the precursors have already been converted into end products, so there were more end products and minimal amounts of precursors present. Indeed Graham et al. (2010) noted that AN levels reach their maximum in leaves at node 11 and older, suggesting full maturity of trichomes. There is a strong correlation between AN content and trichome population (Kapoor et al., 2007; Arsenault et al., 2010a; Graham et al., 2010). The current study shows there is also a correlation between end products and trichome sac size consistent with observations by Maes et al. (2010). At least for AN, the accumulation of end products in mature leaves is linked to the expansion of the trichome sac.

An accumulation of end products was also seen when comparing the metabolite levels of rooted and unrooted shoots. It seems that only when the roots are present are there high levels of end products. The high levels of end products are not accompanied by an increase in transcription of genes in the AN pathway, suggesting that the roots are not regulating the pathway at the level of transcription of the measured artemisinic genes. The trichome sac size of rooted shoots was found

to be significantly larger than those on unrooted shoots. At first glance this seemed to indicate that the presence of roots induced an accumulation of metabolites in the trichome sac causing it to expand. However, the presence of two potent phytohormones, NAA and BAP, in shooting medium may have been confounding this interpretation.

Phytohormones that are known to be involved in plant defense, such as JA, ABA, and SA, increase artemisinic metabolites in *A. annua* and in some cases also alter trichome development (Jing et al., 2009; Guo et al., 2009; Pu et al., 2009; Maes et al., 2010). This is not surprising considering that artemisinin is also thought to be involved in plant defense. It is less clear, however, how other phytohormones, such as auxins (NAA) and cytokinins (BAP), which are involved in plant development, affect the artemisinin pathway.

BAP is known to increase trichome density in many plants, such as maize, tomatoes, poplar, and *A. annua* (Maes and Goossens, 2010; Maes et al., 2011). Indeed, in the young leaves of *A. annua* rooted shoots in this study, BAP increased trichome populations and overall leaf area covered by trichomes, a result consistent with that of Maes et al. (2011). When both phytohormones were added, however, the size of trichomes increased along with their percent of leaf coverage. In contrast, NAA decreased the size of trichomes in young leaves. To our knowledge, not much is known on the effect of auxins, particularly NAA, on trichome development. Although Kim et al. (2007) found that 2,4-dichlorophenoxyacetic acid (2,4-D) can induce development of more glandular trichomes in seedlings of *Tilia amurensis*, in *A. annua*, trichomes on mature leaves seemed less sensitive to either hormone when provided individually. When mature leaves were exposed to both hormones together, however, trichome size declined, suggesting that there was a negative synergistic effect. A summary of these effects on trichomes in developing leaves of rooted shoots is summarized in Figure 3.11.

Cytokinins have been thought to play a role in AN biosynthesis as a result of a study where Sa et al. (2001) constitutively expressed isopentyl transferase in *A. annua*, and both cytokinin and AN levels increased. Maes et al. (2011) later reported that when soil-grown plants were sprayed with BAP, artemisinic metabolites did not increase despite increased populations of glandular trichomes. In our study the young leaves of rooted shoots grown in shooting medium + BAP

showed an increase in the precursors, DHAA and AA, and two of the end products, deoxyAN and AB, but there was no change in AN. In the mature leaves of the same cultures, BAP inhibited AB production by about 60% while also increasing production of DHAA and AA, but without affecting trichome development. Together these results suggest that BAP is shifting artemisinic metabolite concentrations, but without further changes in trichome development (see Fig. 3.11).

Leaf Metabolites Fate		Leaf Trichome Fate		
		Density	Size	Area
NAA	NC NC NC ↑ NC NC ↓ NC ↑↓ **DHAA AN DeoxyAN AA AB**	NC NC	↓ NC	NC NC
BAP	↑↑ NC NC ↑ NC ↑↑ ↑↓ **DHAA AN DeoxyAN AA AB**	↑ NC	NC NC	↑ NC
NAA + BAP	↑↑ NC ↑ ↑ NC ↑↑ ↑↓ **DHAA AN DeoxyAN AA AB**	NC NC	↑ ↓	↑ NC
Increase media salts, vitamins, + sucrose	NC NC NC NC NC NC NC NC NC ↑ **DHAA AN DeoxyAN AA AB**	↑ NC	↓ NC	↓ NC

Figure 3.11. A summary of metabolite and trichome changes in young and mature leaves of rooted shoots in response to changing media constituents. Light arrows, young leaves; dark arrows, mature leaves; nc, no change compared to experimental control. Arrow direction indicates a significant increase or decrease of metabolites of shoots grown in NAA, BAP, and NAA + BAP compared to control in rooting medium. Similarly, arrows direction indicates an increase or decrease in metabolites of shoots grown in rooting medium plus both hormones compared to control in shooting medium to assess the impact of increased salts, vitamins + sucrose in shooting medium.

To our knowledge, not much is known about the effect of auxins on the AN biosynthetic pathway. Although Martinez and Staba (1988) reported that the AN content of unrooted shoots was unaffected by the auxins, 2,4-D and IAA, Woerdenbag et al. (1993) later reported that an optimum concentration of NAA at 0.2 mg L^{-1} yielded the maximum level of AN. In the current study new

growth of cultures grown in rooting medium plus NAA, had decreased AA levels, but increased AB, suggesting that NAA might be driving the conversion of AA to AB. In mature leaves only NAA increases the level of AN when compared to controls. Additionally, AA was not detected in the mature leaves suggesting that it had all been converted to AB. To determine if AN levels could be increased in field-grown plants, we subsequently sprayed mature leaves of potted plants with NAA, but there was no change in AN, or other artemisinic metabolite, content (data not shown).

Reactive oxygen species (ROS) are thought to be involved in the AN biosynthetic pathway, specifically the last step from AA to AB and DHAA to AN or deoxyAN (Fig. 3.1; Brown and Sy, 2007, 2004; Covello, 2008; Mannan et al., 2010). In maize the auxin, indole-3-acetic acid (IAA), was reported to induce ROS in roots (Joo et al., 2001), while NAA induced superoxide radicals (O_2^-) in coleoptiles (Schopfer et al., 2002). Although those auxin levels were at least 20 times greater than used in this study, their results are consistent with our NAA results in mature leaves of *A. annua* suggesting a possible ROS stimulation of AN. Interestingly, the level of AB in these leaves was still low compared to the control without NAA. Considering that AB formation from AA also involves ROS, this suggests that at least in mature leaves there is either a different pathway response to ROS or that the NAA effect is not ROS related.

In contrast to the observed effects of NAA on mature leaves of rooted *in vitro*–grown plants, BAP did not stimulate AN production, nor did it significantly affect the total metabolites (DHAA+deoxyAN+AN) of that pathway. Interestingly, however, BAP did appear to inhibit the AA and AB pathway metabolites by >50%. Taken together with the effects of NAA on mature leaves, it appears that the AA/AB pathway is inhibited by both hormones, but that only NAA stimulates AN production, and only in mature leaves.

While BAP also did not affect glandular trichome population or size in the mature leaves, when both BAP and NAA were present, size decreased suggesting that total artemisinic metabolites decreased. When metabolites were measured, there was a summary overall decline of about 20%, consistent with an equivalent decline in sac size. Furthermore, compared to rooting medium controls, when both NAA and BAP were present there was a major loss in AB (~50%), and an increase in AA and AN/DHAA metabolites. Together these results suggest that in the mature

leaves NAA is likely inhibiting production of AB, but enhancing production of AN. BAP also inhibits AB production in mature leaves, and when the two are present, BAP appears to be the stronger effecter. Like BAP, NAA seems to be regulating the AN biosynthetic pathway directly and not through development of glandular trichomes where artemisinic metabolites are synthesized and stored. Although spraying plants with NAA did not increase AN levels, only one concentration and a single analysis time point were tested, so a broader study may yield different results. Alternatively, NAA was applied via the roots, so AN increase may only occur if roots, and not the shoots, receive the initial signal, a response sequence similar to that with DMSO (Mannan et al., 2010).

An increase in concentration of salts, vitamins, and sucrose also appeared to affect the artemisinin biosynthetic pathway in mature leaves, but mainly by the AB route. When the concentration of these components was increased, as provided in shooting medium, AB also increased. None of the other artemisinic metabolites was affected. Different concentrations of sugars (i.e. sucrose, glucose, and fructose) have been shown to alter production of artemisinic metabolites over time. For example, in unrooted shoots, sucrose levels of 1-3% (w/v) induced optimum production of AN (Woerdenbag et al., 1993; Liu et al., 2003). In *A. annua* seedlings, glucose was shown to stimulate AN production, while fructose inhibited AN production, and as the ratio of glucose to fructose increased, so also did AN production (Wang and Weathers, 2007; Arsenault et al., 2010b), so sugars do affect the AN pathway.

Other nutrients like potassium and nitrogen can also increase artemisinin production. For example, under potassium deficiency, soil-grown *A. annua* plants showed significantly increased production of artemisinin (Ferreira, 2007). Davies et al. (2009) later showed that, although application of potassium between 51 to 153 mg L^{-1} increased biomass, it did not increase artemisinin production. When ammonium nitrate was applied to soil-grown plants as a nitrogen source, artemisinin production increased (Davies et al., 2009). Nitrogen is known to increase leaf biomass, so an increase in AN production is probably the result of more leaves and therefore more trichomes. The current study further supports the idea that factors such as vitamins, salts, and sucrose also play a role in artemisinin production, but not necessarily as regulators.

To determine if the roots of *A. annua* have any regulating effect on AN production that is also not confounded by BAP or NAA, roots were removed from rooted shoots and inoculated into media ± BAP or NAA. Roots were crucial to the continued production of AN in the 3 media tested and this was particularly obvious in the presence of BAP; without roots, BAP actually inhibited AN production. In BAP medium, roots did not re-emerge from the excised shoot, while in NAA medium shoots had developed new roots to replace those that had been excised. Consequently roots were present at harvest and as expected, compared to the NAA-free controls, there was no difference in AN levels. Neither precursors nor end products changed in the shoots ± NAA. Again this was likely because at harvest all shoots formed roots. Unfortunately, no conclusion can be made on whether or not roots play a regulating role in the AN biosynthetic pathway since the controls for both the experiments where roots were removed did not show similar results even when treated the same way. Further experiments are needed in order to clarify this discrepancy.

3.5 Conclusions

In the presence of roots, only NAA is able to induce higher AN production, but only in mature leaves. Additionally, when both NAA and BAP affect a pathway, the effects of BAP always appear to override the effects of NAA. Overall, there was no difference in the amount of total end products of rooted shoots grown with either of these hormones compared to those grown in just rooting medium. Furthermore, an increase in other media constituents showed lower percent leaf surface area occupied by glandular trichomes even though trichome population density increased. Together these results have furthered our understanding of the role of NAA and BAP in glandular trichome development and the production of artemisinin in *A. annua*.

4. Future Work

From this work, there are two possible routes for future studies. First is to examine the effects of growth phytohormones on the AN biosynthetic pathway. Although some work has been done with BAP and GA_3, not a lot is known about how NAA or other more natural auxins affect the AN pathway. In this study, 0.25 µM NAA was found to increase AN production in older leaves of *in vitro* rooted shoots after three weeks. It would be interesting to test other NAA concentrations. Additionally, NAA is a synthetic auxin and therefore does not break down as fast as natural auxins such as IAA. Since there are many other synthetic auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), it is possible that one of them could be used to promote higher AN production than that seen from NAA. The experiments in this study were done *in vitro*, so these results may not mirror responses of soil-grown plants. If soil-grown plants are used for testing different NAA concentrations as well as other types of synthetic auxins, it may lead to a process that could be used to increase AN production in field-grown plants.

The second investigative route would be to determine what in the roots is regulating the production of artemisinic metabolites. This study suggested that the roots play an important role in the AN pathway, but how is still unclear. We hypothesized that there may be a mobile signaling factor traveling from the roots to shoots to regulate production of end products (AN, deoxyAN, and AB). To determine if there is a mobile signaling factor, grafting of the scion of a low AN producing *A. annua* to the rootstock of a high AN producing *A. annua* should be done. After about four weeks, if there is a higher level of end products in the new leaves compared to low-producing non-grafted controls, then this would support the hypothesis that there is a mobile signaling factor and that indeed roots do play a role in AN biosynthesis. Subsequently the chemical nature of that factor would have to be identified.

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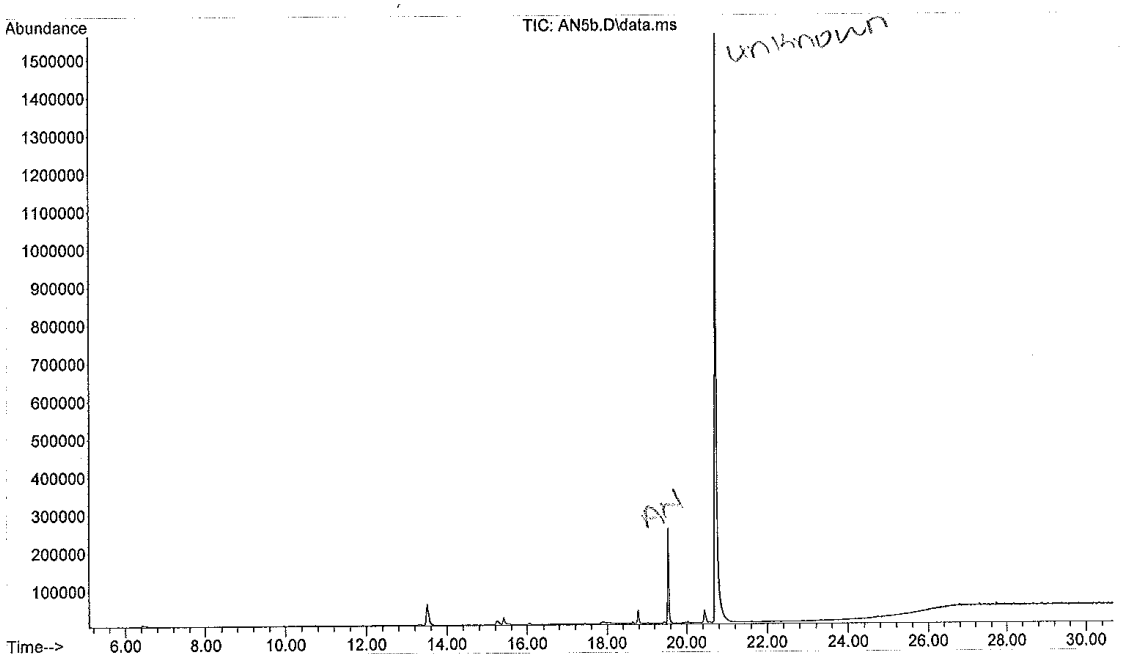
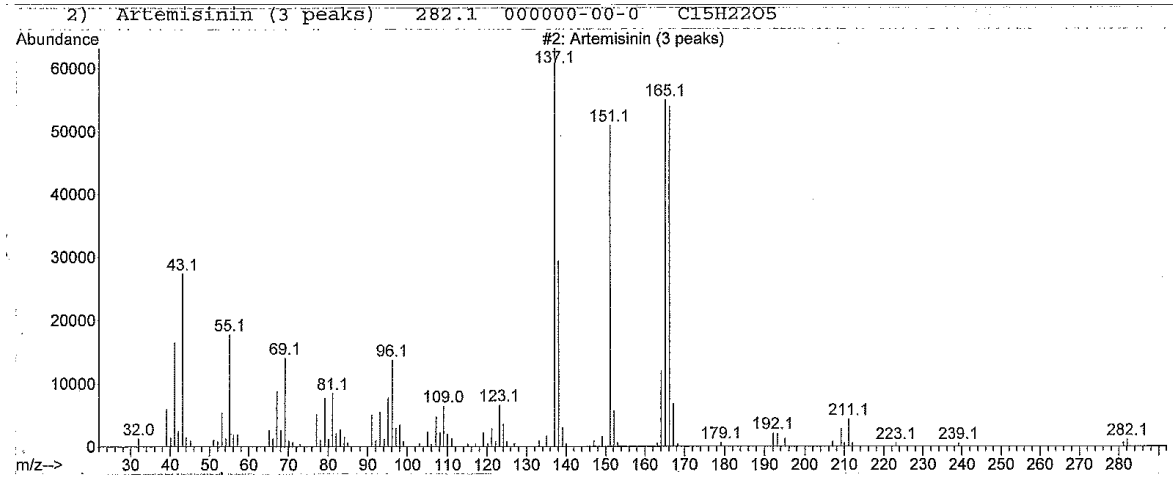
6. Appendix:

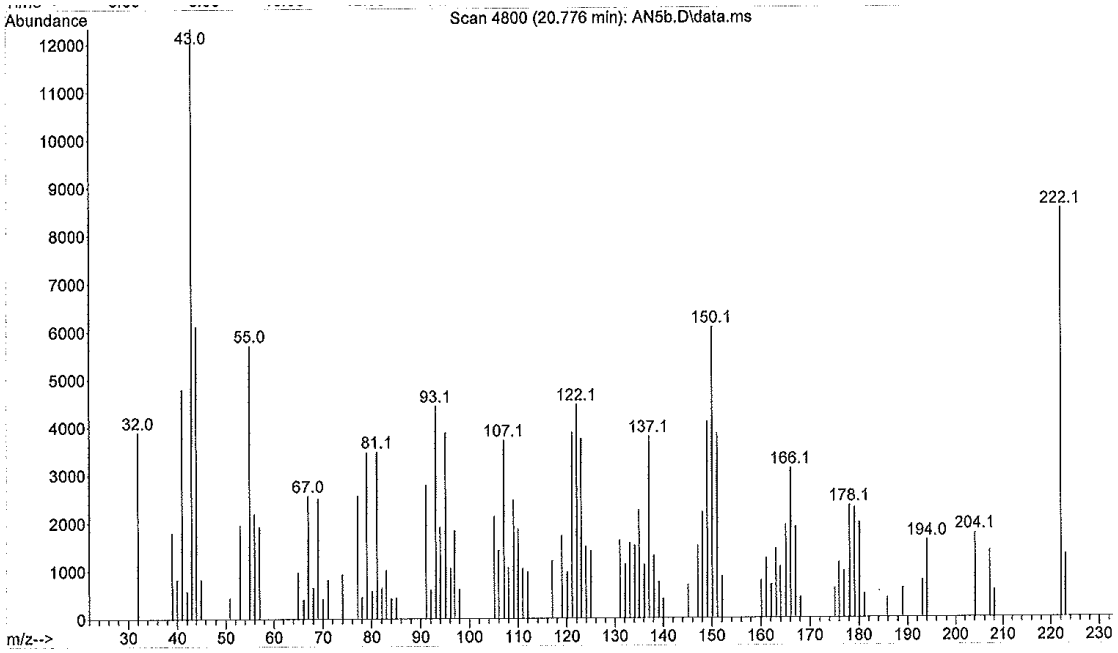
A. Artemisinin (AN) Standard Chromatogram and Mass spectra of AN Peaks



Artemisinin (AN) standard chromatogram



Mass spectrum of AN peak labeled as "AN" in AN standard chromatogram



Mass spectrum of AN peak labeled as "Unknown" in AN standard chromatogram

B. Calculating AN and DeoxyAN in GC-MS Analyses.

Usually, there is a low amount of AN in *in vitro* shoots, therefore a concentration curve was initially produced using AN amounts up to only 100 µg mL^{-1} to ensure the detection of low AN levels. We saw that there was a linear relationship between the AN peak area (AN peak shown in Appendix A) and these AN concentrations. We assumed that 1 µL injection of extract from any tissue, even from soil-grown plants where AN level is high, would not exceed 100 µg mL^{-1} and even if it did, this relationship would still hold at a higher AN concentration. It was later found that this was not the case. Additionally, at the high oven temperature used for AN analysis (described in Materials and Methods 3.2.5), AN can break down into several additional products. In earlier experiments, one of the breakdown products (labeled AN peak in Appendix A) was used to calculate AN concentration in plant extracts, and this proved valid for extracts where AN level was ≤ 100 µg mL^{-1}. Later however, it was found that using the sum of both AN breakdown products (Appendix A, peaks labeled AN and UNK) yielded a more precise AN measurement at high AN concentrations > 100 µg mL^{-1}. Since not much is known about deoxyAN and it is not commercially available, it is unclear if deoxyAN will similarly break down similarly at high temperature. On the other hand, using a pure AB standard (gift of Nancy Acton, Walter Reed Army Institute of Research) we found no AB breakdown products at high temperature.

To calculate the levels of AN and deoxyAN, a concentration curve of the total area of all AN peaks vs. concentration (µg/ ml) was needed. Since no curve was run at the same time as the samples, a composite curve was generated from a series of concentration curves that had been run at different times (from October 2010-May 2011) and was used instead (Fig 6.1). Since at AN concentrations > 100 µg mL^{-1} there was no linear relationship between the total AN area vs. concentration (µg mL^{-1}), a natural log transformation was used. The concentration curve presented below is the ln(total AN area) vs. ln(AN concentration) and the equation for the linear curve fit is y = 1.0727x + 12.786 (Fig. 6.2). This equation was used to calculate the ln(AN concentration) from the total AN peak area taken from the GC/MS, then the AN concentration for 1 µL injection was calculated by taking the inverse of the natural log. An example of this calculation method is presented below.

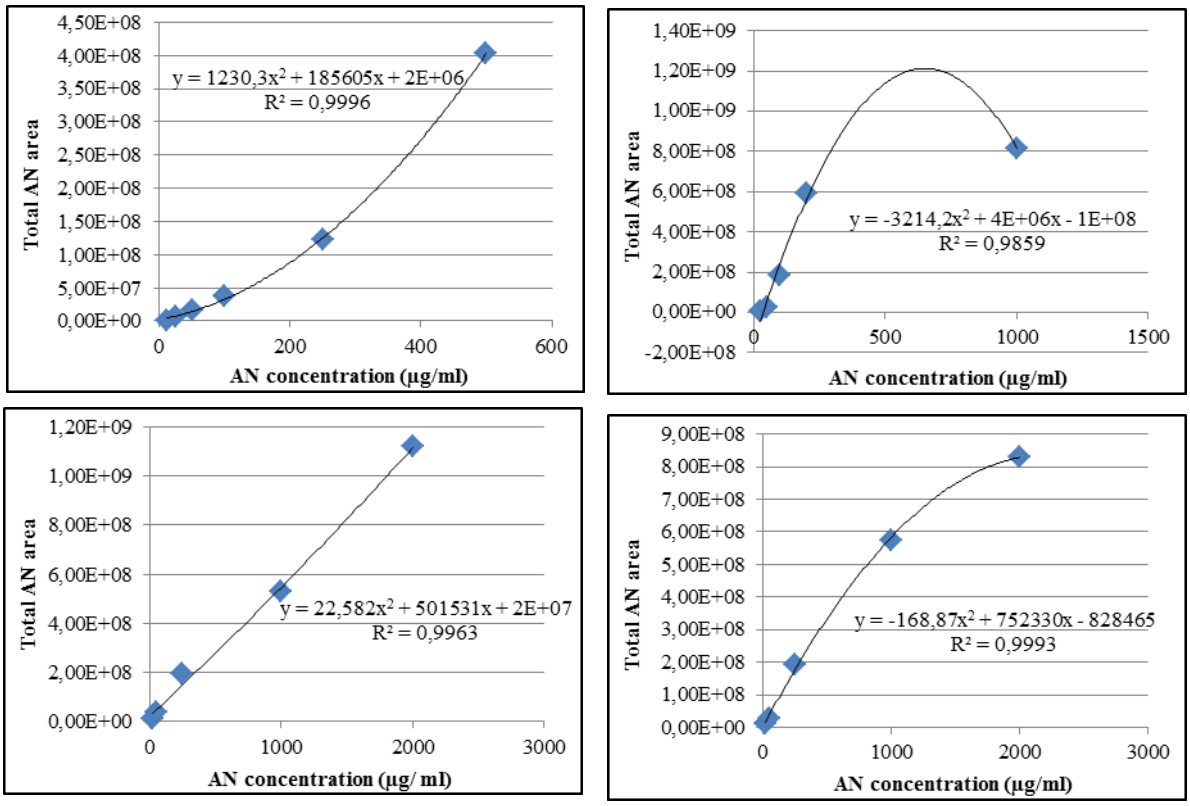


Figure 6.1. Concentration curves of total AN area vs. AN concentration used for natural log transformation.

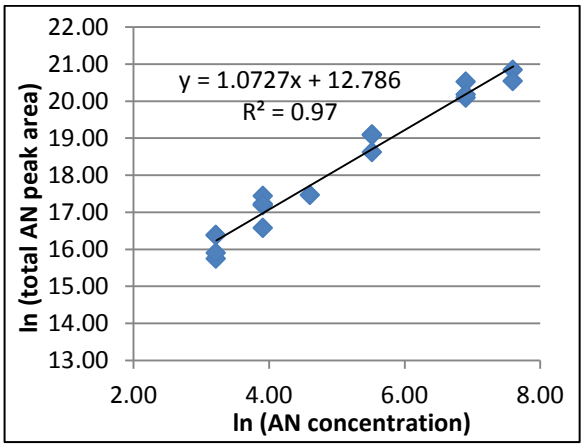


Figure 6.2. Standard curve used for calculation of AN and deoxyAN levels

Example:
y= 1.0727x+12.786 => ln (total area) = 1.0727 [ln (AN concentration)] + 12.786 =>
ln (AN concentration) = [(ln(total area) – 12.786)/1.0727]

Total AN peak area (or peak area of deoxyAN because AN is used to quantitate deoxyAN for 1 µl injection of sample= 100,927,749

ln (AN concentration) = [(ln(100,927,749)-12.786](1.0727)$^{-1}$ = 5.26

AN concentration = e $^{(5.26)}$ = 192 µg mL^{-1}

The total AN amount in 100 µL = 192 µg mL^{-1} * 0.1 mL = 19.2 µg

The fresh weight of this sample is 0.2667 g, therefore the AN concentration per fresh weight = 19.2 µg / 0.2667 g = 72.3 µg g FW^{-1}.

C. **Hydrogen Peroxide Spraying of *A. annua* Plants.**

To determine if, and at what concentration, hydrogen peroxide stimulates the highest level of AN, non-clonal soil-grown *A. annua* (CH strain) soil-grown plants were sprayed with different concentrations of hydrogen peroxide (water, 2.5 mM, 5 mM, and 10 mM) (Fig. 6.3). Five plants were chosen for each concentration and each plant was sprayed with 5 mL of either water or hydrogen peroxide*. After three days, the shoots were harvested and artemisinin was analyzed using HPLC. These data suggest that the highest level of AN (700 µg g DW^{-1}) was achieved using 5 mM hydrogen peroxide. However, subsequent repeats of this experiments by myself and others did not show any significant results and thus, the data shown below were deemed irreproducible.

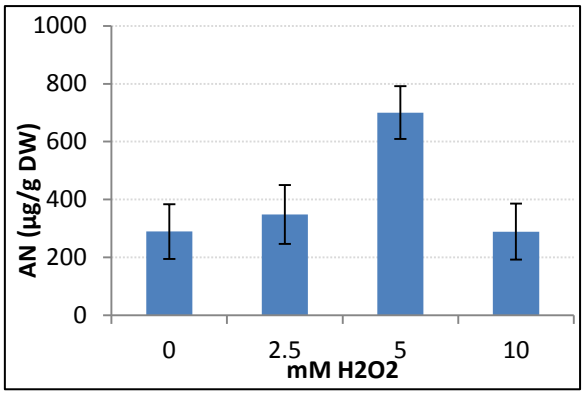


Figure 6.3. Hydrogen peroxide concentration spray (Trial 1)

*Hydrogen peroxide used for this experiment was bought at Walgreens, but they no longer sell this bottle (with a red label on it). The subsequent hydrogen peroxide repeat experiments done by myself, and an undergraduate student, Andrew Keyser, used a different label hydrogen peroxide bottle sold at Walgreens.

D. **Dredging and Root Injury to *A. annua* Plants.**

To determine if wounding the roots of *A. annua* plants would stimulate AN production, non-clonal *A. annua* (CH strain) soil-grown plants were used (Fig. 6.4). On day 0, one set of soil-grown plants was mechanically stressed by stabbing the root zone about 50 times. As a control, another set of soil-grown plants were not mechanically stressed. Plants were watered daily and each day four plants were harvested from each set. Samples were extracted using toluene and analyzed using HPLC. The experiment ran for six days.

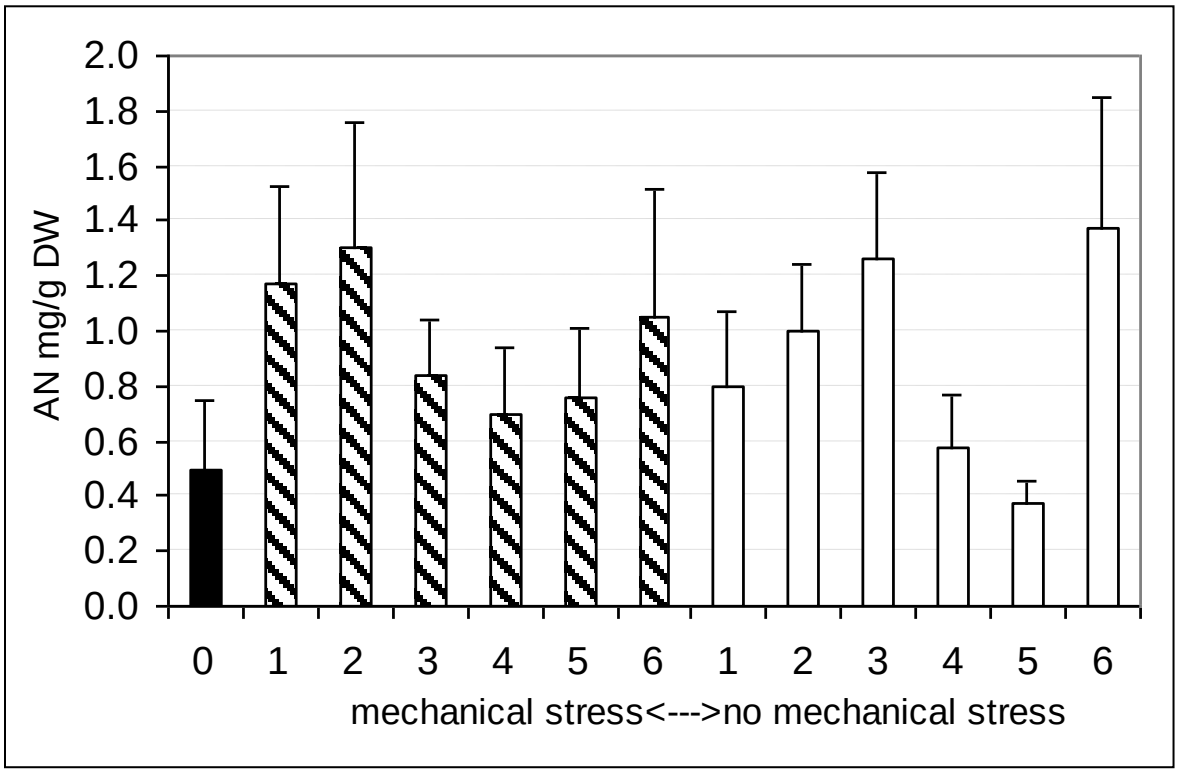


Figure 6.4. Mechanical stress on soil-grown *A. annua*. Numbers represent days. Black, day zero control; hatched, mechanically stressed plants; white, non-mechanically stressed plants.

To determine if constant watering as well as mechanically stressing the plants affect AN production, another experiment was done in which plants were either mechanically stressed or not (M or NM) and were watered daily (prefix W) or just once at on day 0 (Fig. 6.5). The experiment ran for four days (1-4) and each day five plants from each group were harvested and extracted with toluene. Samples were analyzed using HPLC. Although in the data shown above there appeared to be no effect of mechanically stressed roots on AN production in the leaves, a later experiment suggested otherwise (see below). Mechanically stressing the plants by wounding the roots

stimulated AN production with highest AN level on day 2. Watering did not show any significant effect.

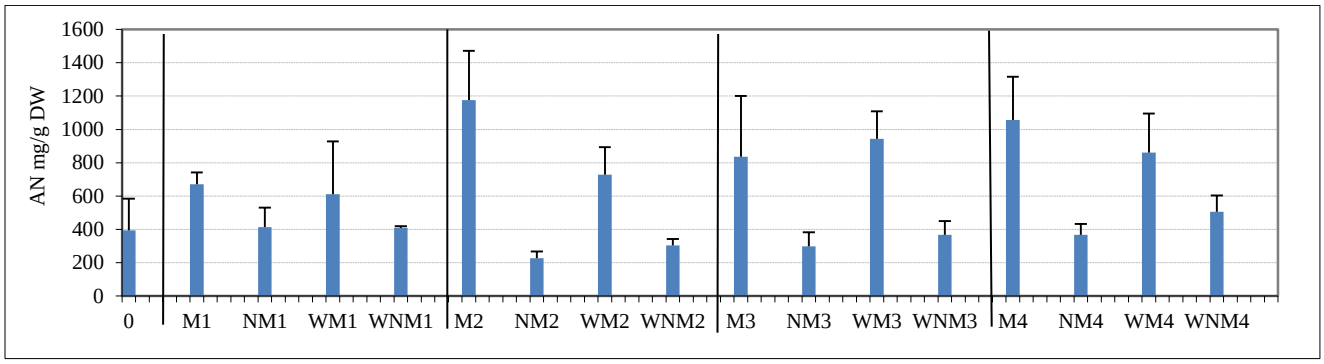


Figure 6.5. Mechanical stress and dredging on soil-grown *A. annua*. Numbers represent days. M, mechanically stressed; NM, not mechanically stressed; W, watered daily.

Recent experiments used rooted shoot cultures of *A. annua* (clone Sam) grown in Magenta boxes where half the cultures had their roots cut with a scalpel, while the other half of the cultures did not (Fig. 6.6). After 48 hrs there was no difference in AN level in shoots from wounded root plants and unwounded rooted plants. Conclusion was that neither peroxide spraying or root wounding yielded any reliably significant increase in AN in shoots.

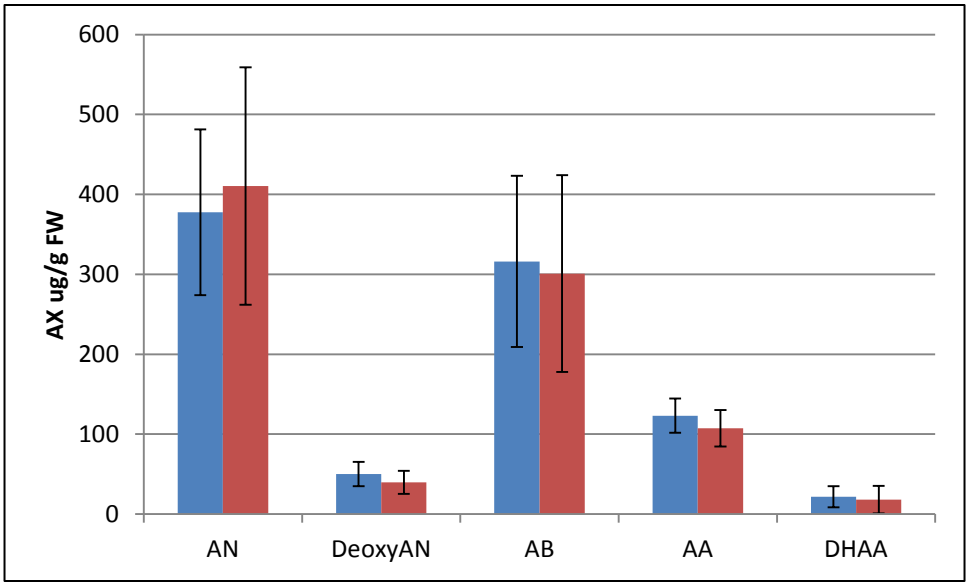


Figure 6.6. Individual artemisinic metabolite levels of *in vitro A. annua* with their roots wounded. Blue, control; red, rooted shoots with their roots wounded; AN, artemisinin; deoxyAN, deoxyartemisinin; AB, arteannuin B; AA, artemisinic acid; DHAA, dihydroartemisinic acid.

E. Spraying NAA on *A. annua* Potted Plants.

Plants of SAM cuttings (July 5th 2011) were transplanted to square pots (July 27th 2011) and eight plants were sprayed with either 10 mL dH_20 or 0.25 uM NAA (both eith pH 5.8 and autoclaved) on August 8th 2011. Additionally, four plants were watered with 0.25 uM NAA in the root zone only or watered daily with dH_20. After three days, all plants were harvested. Young leaves were the first 2 fully open leaves plus all younger tissue. Mature leaves were leaves 9-12 from the apical meristem. Tissues were extracted with pentane and analyzed by GC-MS.

Treatment	**leaf age**	**AN µg/g FW**	**deoxy µg/g FW**	**AB**	**AA µg/g FW**	**DHAA µg/g FW**
SPRAYED	mature	572.61±204.22	117.02±38.78	nd	nd	6.59±3.17
dH_2O	young	197.66±48.58	65.47±15.17	nd	119.33±31.29	1601.36±430.37
NAA	mature	514.11±150.83	108.05±29.54	nd	nd	8.64±3.38
	young	148.95±41.08	48.71±14.08	nd	104.58±43.21	1332.06±442.65
ROOT ZONE	mature	444.10±24.01	94.31±6.93	nd	nd	10.48±1.86
dH_2O	young	142.60±10.86	42.01±2.58	nd	92.74±40.13	1269.19±468.70
NAA	mature	430.22±86.49	90.50±12.68	nd	nd	10.85±4.08
	young	174.30±55.72	55.51±18.99	nd	104.80±34.33	1360.28±435.38

Figure 6.7. Individual artemisinic metabolite levels of potted *A. annua* plants sprayed with NAA. nd = not detected