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Root and Shoot Elicitation in *Artemisia annua*:

Chitosan and Salicylic Acid Effect on Artemisinin and Flavonoid Biosynthesis

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Abstract

Our project analyzed root and shoot elicitation of the anti-malarial drug-producing plant, *Artemisia annua*, using the elicitors chitosan and salicylic acid. We accomplished this by applying chitosan and salicylic acid to the roots and shoots of hydroponically grown *A. annua* and harvesting both the tips of the apical meristem and the third through fifth leaves from the tips at 48 and 96 hours, and 96 and 120 hours, respectively. The antimalarial drug, artemisinin, and flavonoids that act synergistically with the drug were extracted and quantified using GC/MS and an aluminum chloride assay, respectively. We observed higher concentrations of artemisinin in the tips of plants treated with chitosan via the roots indicating chitosan elicits artemisinin biosynthesis and that the roots play an important role in the production of artemisinin.

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Executive Summary

Malaria is a severe and potentially fatal vector-born disease that has affected the world for nearly 4,000 years. In 2010, the World Health Organization (WHO) estimated there were 216 million cases of malaria and more than 655,000 deaths, mainly African children (WHO, 2012). In developing countries, it is the 5th most common infectious disease and the 10th most common cause of death (Mathers and Loncar, 2006). The most common treatment for malaria involves an Artemisinin-based Combination Therapy (ACT). The active drug, artemisinin (AN), is a secondary metabolite produced by the plant *Artemisia annua* L. for defense purposes (WHO 2009). However, due to the high cost of production (Mutabingwa, 2005) and low yield of the active drug, at best approximately 1.5% of the plant leaf mass, (Kindermans et al., 2007), a new method for drug production needs to be found.

Recently, Elfawal et al. (2012) showed that "orally ingested powdered dried leaves of whole plant *A. annua* kills the malaria parasite more effectively that a comparable dose of the pure drug" possibly due to AN working synergistically with six other flavonoids against the malaria parasite. A whole plant therapy has the potential to increase the number of patients treated each year with malaria by reducing the cost of drug production. Whole plant therapy can stimulate the economies of developing nations through cultivating the plant, processing the plant material, and distributing the whole plant therapy among its citizens (Elfawal et al., 2012).

Elicitation is the process of inducing the production of secondary metabolites such as AN or other flavonoids (Taiz and Zeiger, 2010). In this study, the response of AN and flavonoid production to the presence of the elicitors, chitosan and salicylic acid, was studied. The response of the secondary metabolite concentration to application of these elicitors when applied to the shoots and roots was also investigated. Finally, the concentration of the secondary metabolites in the undeveloped shoot tip leaves (hereafter referred to as TIPs) (juvenile leaves) and the 3rd-5th fully expanded leaves from the shoot tip (mature leaves) was compared.

To complete these experiments, *A. annua* (SAM cultivar) was grown hydroponically and either chitosan (CH) or salicylic acid (SA) was applied to the roots or shoots. After 48 and 96 hours (CH treated plants) and 96 and 120 hours (SA treated plants) the TIPs and the 3rd-5th

leaves from the shoot tip were harvested. Artemisinin (AN) and flavonoids (FLVs) were then extracted using methylene chloride and the concentrations were profiled using thin layer chromatography (TLC) and quantified with gas chromatography mass spectrometry (GCMS) and $AlCl_3$ assay, respectively.

Statistical analysis using independent T-test revealed that CH elicitation via the roots produced trends of increased AN, deoxyAN, and FLV concentrations in the shoot tips. Although application of CH to the shoots increased secondary metabolites in juvenile leaves (TIPs), but the results were not statistically significant. The results suggest that juvenile leaves were more responsive to CH especially when applied to the roots.

Analysis of SA treated plant extract samples showed that elicitation via the roots produced trends of increased AN, deoxyAN (AN precursor), and flavonoids in the TIPs. Application of SA to the leaves, however, showed no changes in secondary metabolite concentrations. Mature leaves showed no change in AN or flavonoid concentrations regardless of site of application. SA may have increased secondary flavonoid concentration in TIPs but only when applied to the roots. This study provides further evidence that the roots play an important role in secondary metabolite production in juvenile leaves of *A. annua*.

Chapter 1: Introduction

Malaria is a severe and potentially fatal vector-born disease that has affected the world for nearly 4,000 years. In 2010, the World Health Organization (WHO) estimated there were 216 million cases of malaria and more than 655,000 deaths, mainly African children (WHO, 2012). In developing countries, it is the 5th most common infections disease and the 10th most common cause of death (Mathers and Loncar, 2006). Currently, 40% of the world's population is at risk of malaria. Every 30 seconds, a child dies from malaria. Those children who survive often are inflicted with brain damage and learning impairments (RBM, 2010).

Human malaria is caused by four different protozoan *Plasmodium* parasites*: Plasmodium vivax, P. malariae, P. ovale,* and *P. falciparum* (de Ridder et al., 2008). *P. falciparum* is the most common form of malaria found in sub-Saharan Africa (RBM, 2010). These parasites are transmitted after a human is bitten by an infected female *Anopheles* mosquito. Once in the blood stream, the parasite will first infect and reproduce in the liver, then continue on by infecting red blood cells. When the disease progresses to this severity, it is referred to as 'blood stage'. Blood stage parasites are responsible for the debilitating symptoms of malaria. Certain blood stage parasites, known as "gametocytes" are able to be ingested by female *Anopeles* mosquitos during a blood meal and begin another growth cycle. Between 1—18 days after ingestion, the parasites will localize to the mosquito's saliva in order to infect its next human victim (CDC, 2010). Symptoms of malaria usually appear 9-14 days after the individual has been bitten. These symptoms include vomiting, headache, fever, as well as other flu-like symptoms. If there is a lack of available treatment or if malarial drug resistance to the current treatment is present, the disease will progress and become life threatening. The lethality of the disease results from the rapidity of the destruction of red blood cells, which disintegrate and clog capillaries that lead to the brain or other vital organs (RBM, 2010).

The most common treatment for malaria involves an Artemisinin-based Combination Therapy (ACT). The active drug, artemisinin (AN), comes from the plant *Artemisia annua* L. (Figure 1), also known as sweet wormwood (WHO, 2009). However, due to the high cost of production (Mutabingwa, 2005) and low yield of the active drug, at best approximately 1.5% of the plant leaf mass, (Kindermans et al., 2007), a new method for drug production needs to be

found. Recently, it was proposed that a whole plant therapy may be effective as a new treatment for malaria (Weathers et al., 2011). Although the mechanisms have yet to be fully elucidated, it is thought that AN works synergistically with flavonoids, which are secondary metabolites found in the plant, to create a very effective herbal therapy. In support of this whole plant therapy is the fact that dried *A. annua* leaves yield a higher concentration of AN in the blood stream that when compared to the same amount of the pure drug. In addition, oral consumption of the whole plant seems to provide more bioavailability of AN than by synthesizing the pure drug (Weathers et al., 2011). In addition, Elfawal et al. (2012) recently showed that whole plant *A. annua* was at least five fold more effective than pure AN at reducing parasitemia in mice; the authors call whole plant therapy, pACT, plant delivered artemisinin combination therapy.

Figure 1: *Artemisia annua* plant (SAM cultivar).

This project repeats previously reported elicitation experiments that were used to increase artemisinin production in *A. annua* by means of the application of salicylic acid and chitosan on the shoots. However, several recent studies suggest that roots may also be a potent

site for elicitation AN production in the shoots (Ferreira and Janick, 1996; Mannen et al., 2010; Nguyen et al., 2012). This project tests the effect of salicylic acid and chitosan elicitation via the shoots and roots on the production of both AN and flavonoids in the shoots of *A. annua*.

Chapter 2: Literature Review

Artemisinin (AN) and *Artemisia annua* has been extensively studied for its potential medical properties. In this chapter, the application and biosynthesis of AN are summarized. The *in vivo* synergistic interactions of AN with six other flavonoids are also explained. The process of elicitation of AN in *A. annua* is discussed along with previous research on *A. annua* to elicit the production of AN.

2.1 Significance of artemisinin

The use of *A. annua* for medicinal purposes dates back to 168 B.CE. A record of *A. annua* being used as an ingredient for treatment of 52 diseases was found in the Manwangdui tomb of the Han dynasty. The "Handbook of Prescription for Emergency Treatment" written between 281- 340 C.E. by Ge Hong, explains that tea-brewed *A. annua* leaves can be used to treat fever and chills. It was not until 1972 that the active drug, artemisinin (AN), was identified as the anti-malarial element of *A. annua* (Effereth, 2009). It is believed that the anti-malarial effect of whole plant treatment, such as Chinese infusion tea, is attributed to the synergistic effects of AN with possibly six other flavonoids

The most common treatment for malaria involves an Artemisinin-based Combination Therapy (ACT), e.g. artemether plus lumefantrine. Using ACT reduces the risk of resistance to the active drug, AN (WHO, 2009). AN is a secondary metabolite produced by *A. annua* also known as sweet wormwood (see Figure 1). *A. annua* is classified as a flowering plant, belonging to the class Magnoliopsida and the Asteraceae family. *A. annua* grows to 2m in height and can be found in temperate regions of China, Vietnam, India, Russia, East Africa, the United States, and Brazil (Bhakuni et al., 1988).

Today, ATCs are a promising treatment option for the management of malaria due to the low emergence rate of resistance. However, combination therapies, although more effective in the treatment of the disease, can cost up to twenty times more than monotherapies (Mutabingwa, 2005), where only a single drug is used. Due to the major cost required to treat malaria with combination therapies, many developing nations are hesitant to adopt ACTs without assistance funding from sponsoring agencies such as the World Health Organization (Mutabingwa, 2005). In addition to the high cost required for production, low yields of the active drug are another major drawback to ATCs. *A. annua* yields 0.5-1.2% dry weight (DW)

artemisinin, which means that in one hectare *A. annua* will only produce about 6-14 kgs of the drug (Guthmann et al., 2008). Due to the price of production and the low yields of the active drug, research has focused on new methods for producing artemisinin.

Because of the demand and limited supply of AN, researchers have searched for a way to mass produce AN for a lower cost than current the chemical synthesis. Zeng et al. (2008) described engineering of transgenic microbes such as *Saccharomyces cerevisiae* and *Escherichia coli* to express the AN biosynthetic genes. The complete biosynthetic pathway of AN has not yet been achieved, but is nearly complete with synthesis to dihydroartemisinic acid…one step from AN (Westfall et al, 2013). Transgenic yeast can produce equivalent amounts of *A. annua* but in a significantly shorter amount of time than *A. annua* (5 days verses several months), a potential economic advantage (Zeng et al., 2008). *E. coli* has the potential to produce AN at rapid growth rates but due to the difficulty of reassembling one of the key enzymes, a cytochrome P450, producing AN via *E. coli* remains challenging (Zeng et al., 2008). Dietrich et al. (2009) created a novel approach for "the selective oxidation of amorphadiene, yielding artemisinic-11S,12-epoxide." *Bacillus megaterium* was used to produce aretemisininc-11S,12-expoxide, which then can be chemically synthesized with high yield to dihydroartemisinic acid and for a one step synthesis to artemisinin (Dietrich et al., 2009).

Recently, it was hypothesized that whole plant therapies (pACT) may be more effective than treatment with only the pure drug. When mice were fed AN, it was shown that AN was more readily absorbed into the blood stream when the dried leaves of the whole plant were ingested. At least twice as much time is needed for pure AN to be absorbed into the blood stream than from absorption after whole plant consumption. Additionally, in order for equal amounts of AN to be absorbed into the blood stream the amount of pure drug needed is 45 times greater than from the dried *A. annua* plant material. (Weathers et al., 2011).

Elfawal et al. (2012) showed that "orally ingested powdered dried leaves of whole plant *A. annua* kills the malaria parasite more effectively that a comparable dose of the pure drug". Therefore, pACT has the potential to increase the number of patients treated each year with malaria by reducing the cost of drug production and by providing more doses per acre because the associated losses from extraction are eliminated. Additionally, pACT can stimulate the economies of developing nations by locally cultivating the plant, processing the plant material, and distributing the pACT among its citizens (Elfawal et al., 2012).

2.1.1 Artemisinin biosynthesis

Artemisinin is a secondary metabolite produced by and stored in the glandular trichomes of *A. annua* (Bouwmeester et al.,1999; Aftab et al., 2011). Secondary metabolites are organic compounds produced by plants that do not directly play a role in the growth, reproduction, or photosynthesis of the plant. Secondary metabolites have many ecological functions such as acting as the plant's defense system against physical damage, chemicals, and parasites. In addition they may attract pollinators or seed-dispersing animals via color, taste, or odor. Secondary metabolites can be categorized into three groups: nitrogen containing compounds, phenolics, and terpenes (Taiz and Zeiger, 2010). AN is a sesquitepene lactone (see Figure 2).

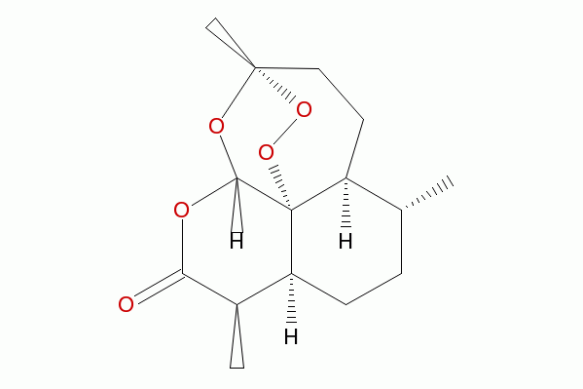


Figure 2: Chemical structure of artemisinin.

Three isopentenyl diphosphate (IPP) precursor molecules are condensed and oxidized to produce AN (Bouwmeester et al., 1999; Towler and Weathers, 2007). IPP is produced through two pathways: the cytosolic mevalonate (MVA) pathway or the plastidic non-mevalonate (MEP) pathway. Farnesyl diphosphate (FDP) is produced when FDP synthase (FPD) catalyzes the condensation of three IPP precursor molecules (Figure 3). Next, amorpha-4,11-diene is catalyzed by amorphadiene synthase (ADS), a sesquiterpene cyclase (Bouwmeester et al., 1999; Kim et al., 2008; Wallaart et al., 2001). The next three reactions are the oxidation of amorpha-4,11-diene to artemisinic aldehyde and to artemisinic acid (AA) and are catalyzed by a cytochrome P450, CYP71AV1 (CYP) (Teoh et al., 2006). AA is then converted to dihydroartemisinic aldehyde by a double-bond reductase (Dbr2). Dihydroartemisinic aldehyde is thought to be the precursor to dihydroartemisinic acid (DHAA) (Zhang et al., 2008).

In vitro, the conversion of DHAA to AN occurs as a photo-oxidative reaction, however, it has not been fully verified if this also occurs *in vivo.* What is known, is that the conversion reaction requires the addition of three oxygen atoms and the development of an endoperoxide pharmacophore of AN. It is not completely understood how the enzymes are controlled nor how the levels of AN and its precursors are influenced (Mercer et al., 2007).

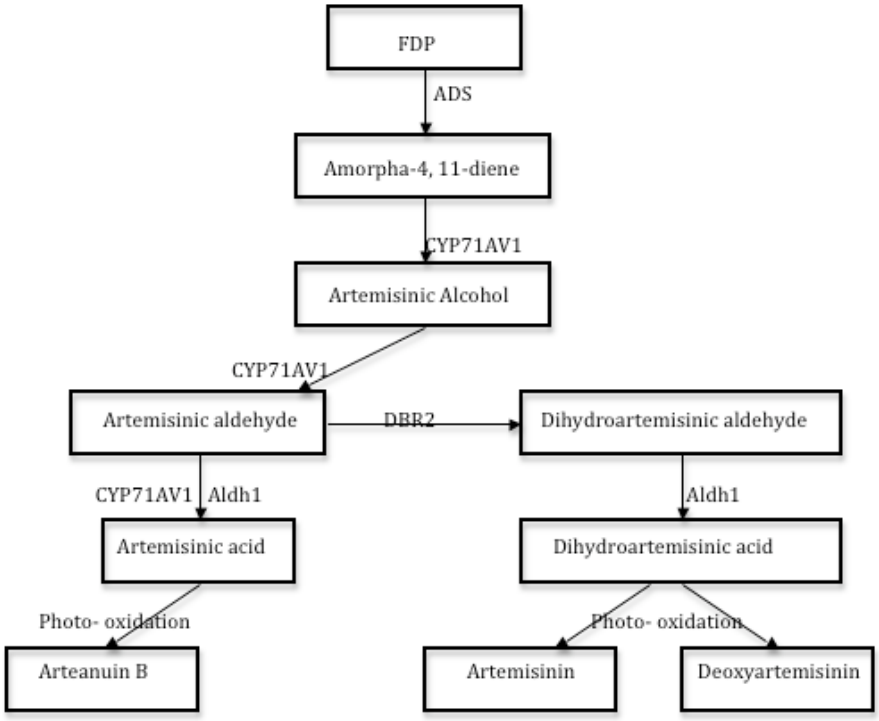


Figure 3: Biosynthesis of AN in *A. annua*.

2.2 Significant of flavonoids

Flavonoids are a class of phenolic compounds. Phenolic or polyphenol compounds mainly include tannins and flavonoids. A plant synthesizes these phenolic compounds to respond to stress conditions such as pathogens, wounding, and UV damage (Sakihama et al., 2002). Flavonoids are subcategorized based on their structure: flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones. Flavonoids are also important compounds because of their benefits to human health; these compounds have antiviral, anti-allergic, anti-platelet, anti-inflammatory, antitumor, and antioxidant activities (Buhler and

Miranda, 2000). Over 4,000 flavonoids have been identified, and most of them occur in fruits, vegetables, and beverages, including coffee, beer, wine, and fruit drinks (Buhler and Miranda, 2000).

2.2.1 Flavonoid biosynthesis

The amino acid phenylalanine (Phe) and malonyl-coenzyme A (CoA), which is produced via the fatty acid pathway, are precursors for flavonoids (Winkel-Shirley, 2001). Chalcone is the first true product of the flavonoid pathway and is derived from phenylalanine and malonyl-CoA by chalcone synthase. The flavonoid pathway is depicted in Figure 4 (adapted from (Rangarajan et al., 2004)). Chalcone is then isomerized to flavonone by chalcone isomerase. Flavonone is either hydroxylated to dihydroflavonol, responsible for the production of anthocyanins and flavonol glycosides, or converted to flavones or isoflavones (Rangarajan et al., 2004).

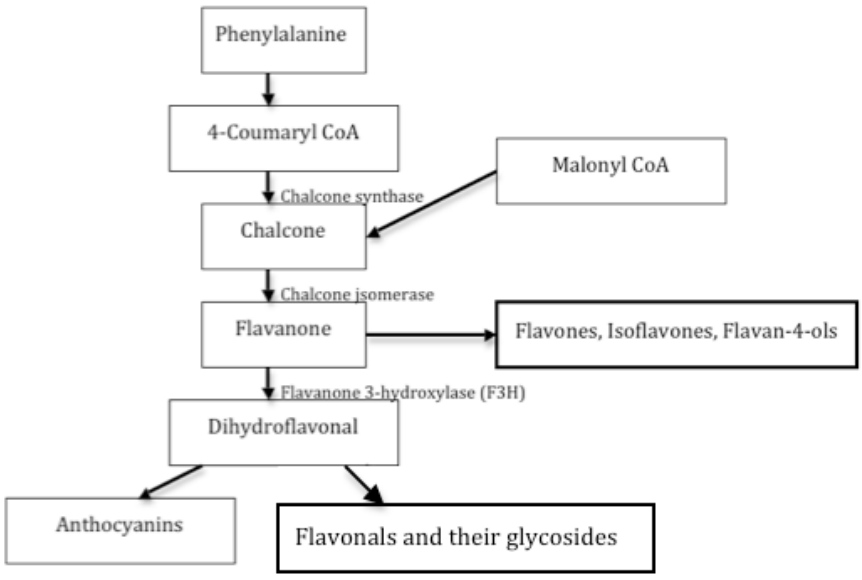


Figure 4: Chalcone is the first true product of flavonoid synthesis.
Phenylalanine and Malonyl CoA are precursors to flavonoids.

2.2.2 Flavonoids in *A. annua* and synergy with artemisinin

There are five main phenolic groups found in *A. annua*: coumarins, flavones, flavonols, phenolic acids, and miscellaneous, (Figure 5) (Sakihama, et al., 2002). The main flavonoids that have shown synergistic properties with AN are classified as either flavones or flavonols. These flavonoids are: cirsilineol, eupatorin, artemetin, chrysosplenol-D, casticin, chrysoplenetin, quercetin, and rutin (Liu et al., 1992; Figure 5).

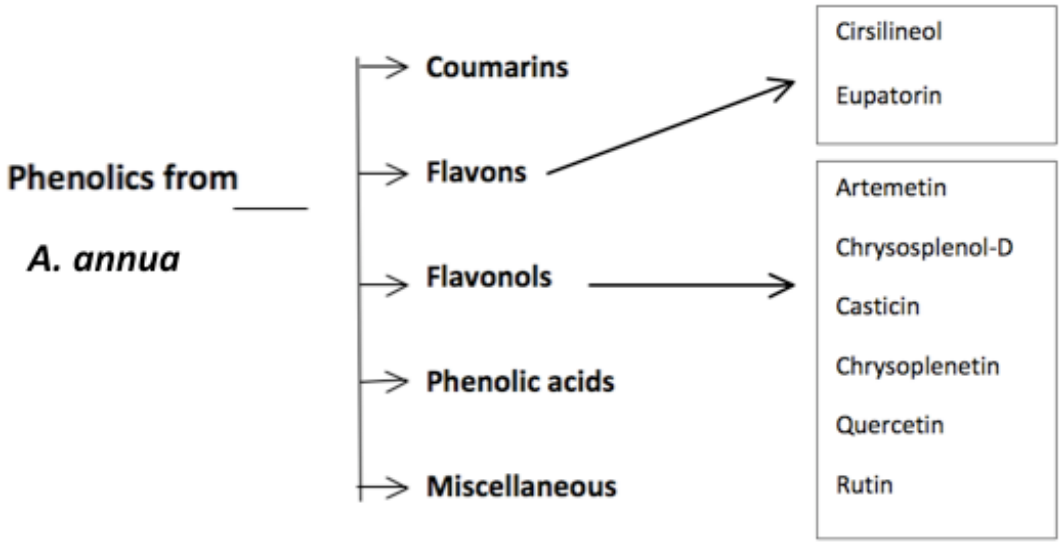


Figure 5: Classification of flavonoids known to work synergistically with AN.

A. annua has many different cultivars, which contain varying levels of AN and flavonoids, some examples are shown in Table 1 (Weathers and Towler, 2012). The concentration of AN in FLV5 is higher than Clone 15, but the concentration of artemetin and casticin are both lower. The SAM cultivar has a higher concentration of AN, artemetin, and casticin than both FLV5 and Clone 15 (Weathers and Towler, 2012).

Table 1: AN, artemetin, and casticin concentrations of various cultivars (Weathers & Towler, 2012).

Cultivar	Artemisinin (mg/g DW)	Artemetin (mg/g DW)	Casticin (mg/g DW)
FLV5	14.89	0.210	0.410
Clone 15	5.71	0.080	0.211
SAM	14.89	0.210	0.410

A. annua contains many flavonoids, but six specific flavonoids artemetin (ART), casticin (CAS), chrysoplenetin (CRY), chrysoplenol-D (CRYD), cirsilineol (CIR), and eupatorin (EUP) (Figure 6), have been shown *in vitro* to work synergistically with AN and work therapeutic against malaria (Liu et al., 1992).

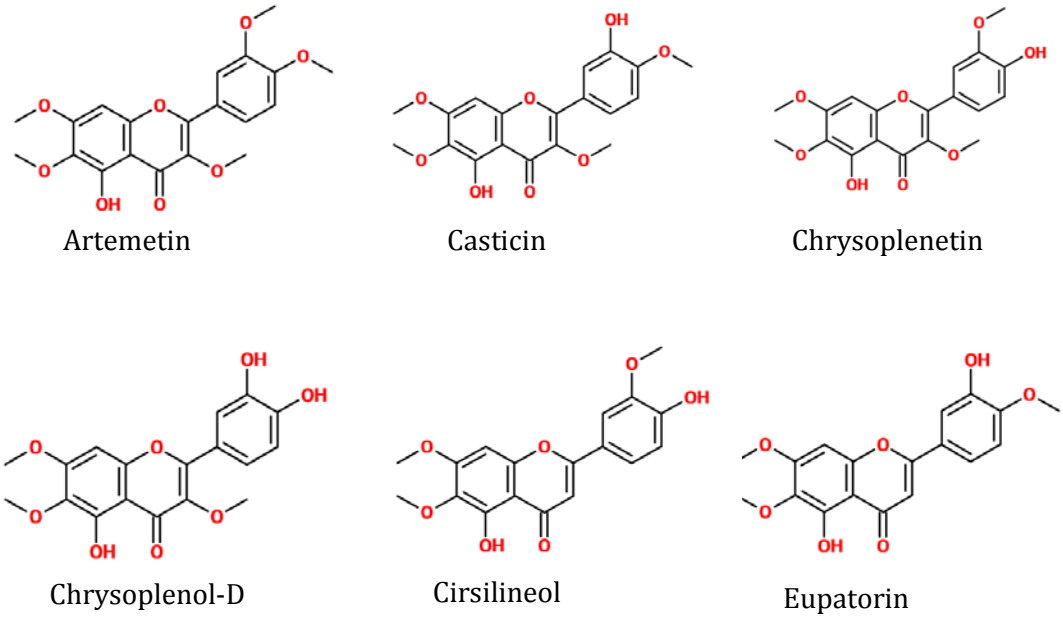


Figure 6: Structure of the six main synergistic flavonoids in *A. annua*.

Liu et al. (1992) found that when combined together with any of these six flavonoids, the required dosage to decrease the parasite concentration by 50% (IC_{50}) was 10-50% less than with just AN alone, as depicted in Table 2.

Table 2: Comparison of the IC_{50} of AN and flavonoids (Liu et. al., 1992).

Compound	IC_{50}		Decrease of AN IC_{50}
	Flavonoid (M)	AN + Flavonoid at 5 µM (M)	
AN	---	3.3×10^{-8}	---
Artemetin	2.6×10^{-5}	2.6×10^{-8}	21%
Casticin	2.4×10^{-5}	2.6×10^{-8}	21%
Chrysoplenetin	2.3×10^{-5}	2.25×10^{-8}	32%
Chrysoplenol-D	3.2×10^{-5}	1.5×10^{-8}	54%
Cirsilineol	3.6×10^{-5}	1.6×10^{-8}	52%
Eupatorin	6.5×10^{-5}	3.0×10^{-8}	9.1%

As seen in Table 2, each of the six flavonoids listed decreased the IC_{50} of artemisinin against *P. falciparum,* suggesting a synergistic effect occurs between artemisinin and the listed flavonoids. The IC_{50} of artemisinin against *P. falciparum* when combined with artemetin and casticin decreased by about 20%, chrysoplenetin by 30%, chrysosplenol-D and cirsilineol by 50%, and eupatorin by 10% (Liu et al., 1992).

2.3 Elicitation of artemisinin and flavonoids

Plants have evolved a variety of defense strategies against insect herbivory and microbial pathogens. There are two defense mechanisms present in the plant. Those that are continually expressed are termed 'constitutive defenses'. However, some defense mechanisms are only activated after damage occurs; these are termed elicited or 'induced defenses'. Insect herbivores can be classified into three categories: phloem feeders, cell content feeders, and chewing insects. While each damages the plant to varying degrees, the plant will respond to the damage with both a response to the physical wounding, and a response to the recognition

of specific insect-produced compounds. Compounds produced by the insects are known as elicitors (Taiz and Zeigers, 2010).

Elicitors are types of chemicals and biofactors that have the ability to induce physiological changes in the target organism. Elicitors in plants refer to chemicals that will induce physiological and morphological reactions, resulting in the accumulation of secondary metabolites. Examples of elicitors include the signaling molecules salicylic acid, jasmonic acid, and ethylene. In addition, components of fungal or plant cells walls, bacterial viruses, and herbivore constituents can all induce elicitor activity; such elicitors include chitosan, ß-cyclodextrins, and boron (Zhao et al., 2005).

Elicitors can act as avirulant determinators of a plant's defense system by imitating gene-for-gene resistance in a plant's native immunity. Plant resistance genes allocate resistance by corresponding avirulence (avr) genes through a series of recognition events to a pathogen. Plant receptors, such as proteins of the plasma membrane, specifically the 'R protein', or certain proteins in the cytoplasm, must recognize the avirulence determinants in order to initiate signaling pathways that ultimately result with the synthesis of pathogenesis-related proteins or defense secondary metabolites (Zhao et al., 2005).

The first step in the elicitor signal transduction pathway is signal perception of the elicitor molecule by the corresponding receptor of the plant plasma membrane. Following signals stimulated by elicitors, plant receptors become activated initiating a cascade of effectors including: activation of ion channels, G-proteins, and protein kinases. These activated effectors further the signal transduction pathways by continuing the elicitation response to second messengers, which result in the amplification of the signal to additional downstream reactions (Ebel and Mithöfer 1998; Blume et al., 2000). G-protein coupling with elicitors may aid in the activation of ion channels and regulate ROS production (Roos et al., 1998); (Munnik and Meijer, 2001); (Kawasaki et al., 1999). Ion fluxes are an immediate response of the presence of elicitors. These fluxes can be observed 5 minutes after elicitor exposure. Calcium ion [Ca^{2+}] fluxes, when induced by an elicitor, are important for production of secondary metabolites (Smith, 1994). Calcium ions regulate secondary messengers such as phosphatic acid (PA), inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Meijer and Munnik, 2001). Also resulting from these ion fluxes is cytoplasmic acidification, which leads to oxidative bursts and biosynthesis of secondary metabolites (Sakano, 2001). Reactive oxygen species

(ROS) induce H_2O_2, O_2^-, and OH^- that are generated by NADPH oxidase, apoplastic peroxidase, and other oxidases lcoated in mitochondria, chloroplasts and peroxisimes. ROS aids in the reinforcement of the cell wall, defensive gene activation, hypersensitive cell death, and defensive compound induction (Levine et al., 1994). In certain plant species, ROS is sufficient for the accumulation of secondary metabolites while in others, hydrogen peroxide (H_2O_2) mediates the production of these defensive compounds (Degousee et al., 1994; Guo et al., 1998). An efficient defense against the elicitor is established by signal transduction pathways that are a culmination of sequential reactions consisting of parallel and cross-linking signals resulting in different target responses (Boller, 1995; Zhao et al., 2005).

It is believed that all signal transduction pathways lead to production of various transcription factors regulating the expression of genes involved in the synthesis of secondary metabolites. The activation of these transcription factors can be directly or indirectly stimulated by the elicitor, controlled by other transcription factors, or synthesized from protein- protein interactions, phosphorylation, and dephosphorylation (Hahlbrock et al., 2003).

Some elicitors produce synergistic effects on the production of secondary metabolites. When endogenous (plant-derived) elicitors and exogenous (microbe-derived) elicitors are applied together or two different exogenous elicitors are applied simultaneously, such synergistic effects are most often seen. For example, chitin can enhance methyl jasmonate-induced production of paclitaxel in *T. Canadensis* (Linden and Phisalaphong, 2000). Combination of the endogenous elicitor cerebroside sphingolipid with the exogenous elicitor *N*-acetylchitohexose results in the synergistic amplification of phytoelexin production in rice cell cultures (Umemura et al., 2002).

2.3.1 Other elicitation in *A. annua*

Although mechanical or physical wounding of a plant will result in similar elicitation responses, compounds present in insect saliva often enhance the stimulus signal beyond that of just physical wounding. Elicitor compounds in insect saliva can initiate signal transduction pathways so that plant defense responses, even in distant regions from the initial insult, can prepare for damage (Taiz and Zeigers, 2010).

Jasmonic acid induced elicitation

The octadecanoid pathway is a major signaling pathway involved in many plant defenses against insect herbivory. This particular pathway leads to the production of the plant hormone jasmonic acid (JA). JA levels increase in response to insect damage and initiate the production of proteins involved in defense mechanisms. JA is synthesized from linolenic acid, which is released from the plant membrane lipids. The first three steps of this process occur in the chloroplast forming the intermediate 12-oxo-phyodienoic acid. From the chloroplast, 12-oxo-phyodienoic acid is then transported to the peroxisome where enzymes involved in the β-oxidation pathway complete the conversion to JA (Taiz and Zeiger, 2010). Jasmonic acid can induce transcription of host genes involved in defense mechanisms, such as genes that encode enzymes in the biosynthetic pathways of secondary metabolites (Taiz and Zeiger, 2010). The conserved signaling mechanism of JA resembles those of other plant hormones such as auxin and gibberellins, which can result in the increased production of AN (Katsir et al., 2008). Others also have shown that JA induces AN synthesis and production in *A. annua* (Maes et al. 2010).

Boron induced elicitation in *A. annua*

Boron is mildly toxic to *A. annua*, and can stimulate AN production by inducing stress. Aftab et al. (2012) applied various concentrations of boric acid to the soil before planting *A. annua* (Aftab et al., 2012). The results suggested that 1 mM of boric acid added to the soil increased the AN concentration 27.2% when compared to the control after 150 days of growth. Metrics were established to test how plant health was affected. When 1 mM boric acid was applied as a soil drench, plant shoot height and fresh and dry weights were all slightly reduced when compared to the healthy controls. Although boric acid may be a moderately effective elicitor, the negative effects that this compound has on plants, as well as its potential hazardous effects on the surrounding environment, suggest that boric acid not be used as an elicitor.

ß –cyclodextrins induced elicitation in *A. annua*

ß-cyclodextrins are a non-reducing cyclic oligomer of glucose (Figure 10) produced by the microbial enzyme clyclodextrin glycosyl transferase. Durante et al. (2011) compared the effect of ß-cyclodextrins ± methyl jasmonate on AN production in *A. annua* suspension cell

cultures. The presence of ß-cyclodextrins significantly increased AN levels in the cells determined with high performance liquid chromatography (HPLC). Furthermore, in the presence of both methyl jasmonate (100 µM) and 50mM ß-cyclodextrins, AN levels were even higher, up to 3-fold higher than controls. *CYP71AV1* expression was also enhanced by ß-cyclodextrins and methyl jasmonate (Durante et al., 2011). However, the data in this study are disputable. Because AN is produced and stored in the trichomes of *A. annua* (Bouwmeester et al., 1999; Covello et al., 2007), it is debatable whether a single cell can produce the chemical. Additionally, HPLC has been shown to not distinguish between AN and deoxyartemisinin (deoxyAN), which can result in the overestimation of AN concentrations (Smith et al., 2010).

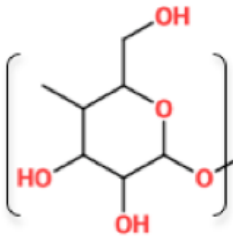


Figure 7: Non-reducing cyclic oligomer of glucose.

Arbuscular mycorrhizal induced elicitation in *A. annua*

Arbuscular mycorrhiza (AM) can enhance production of AN in *A. annua* (Kapoor et al., (2007). AM are fungi that form beneficial association with plant roots, but can also initiate the plant defense system. Kapoor et al. (2007) inoculated microplots ($10m^2$) with one of two species of AM (*Glomus macrocarpum* or *fasciculatum)* before transplanting *A. annua* into the field, and when compared to the controls, 12 weeks later AN production was significantly enhanced in plants grown in plots where either *G. macrocarpum* or *G. fasciculatum* was present. It is possible that chitosan from the fungal symbionts stimulate AN production in the shoots.

2.3.2 Chitosan induced elicitation

Chitosan has been previously shown to elicit AN production after application to leaves of *A. annua* plants (Lei et al., 2011). Chitosan, derived from chitin, is a polymer of β-1,4-

glucosamine residues (Figure 8). Chitin generally is present in the cell walls of fungi and insects and induces the production of secondary metabolites including AN (Amborabé et al., 2008).

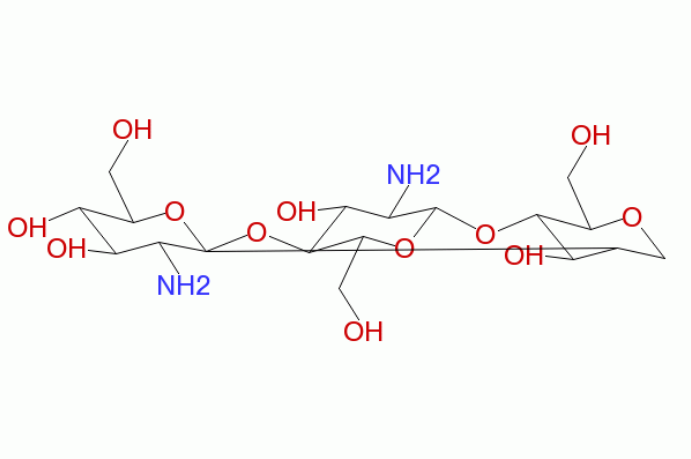


Figure 8: Chitosan

There are several different classes of elicitors that can simulate the effect of fungal elicitors. These classes include polysaccharides and oligosaccarides such as chitin and chitosan (a chitin derivative), xyloglucans, laminarin and other β -glucans. Saccharide elicitors have been extensively studied in several types of plant cells that recognize the elicitor due to similarities within their receptor binding properties (Ito et al., 1997; Okada et al., 2002; Shibuya et al., 2002).

Chitin oligosaccharides induce plant responses though sequential reactions including calcium (Ca^{2+}) ion fluxes, reactive oxygen species (ROS) generation, medium alkalinization, defense gene expression, and phytoalexin biosynthesis (Umemoto et al., 1997; Day et al., 2000; Fliegmann et al., 2003). Although the exact mechanism of how the elicitor triggers ion transport into plant cells is not known, it is clear that ion fluxes, including the influx of Ca^{2+}, can be observed in the earliest responses of plant cells to avirulent pathogen or elicitors (Trewavas and Malhó, 1998; White and Broadley, 2003). Measurements have shown that within two to five minutes of elicitor exposure, elicitors can induce cystosolic calcium $[Ca^{2+}]_{cyt}$ to increase over a hundred fold, spiking from initial resting levels of 50-100nM to 1-5 µM. After the first initial influx, oligosaccharides (chitin and chitosan) elicitors continue to induce a second strong, but brief, increase in cystosolic calcium ion levels. The first oligosaccharide-induced $[Ca^{2+}]_{cyt}$ peak is cause by the influx of extracellular Ca^{2+}, while the second peak is induced by

PLC (phospholipase C) activation and the release of IP_3-release Ca^{2+} from intracellular calcium stores within plant cell organelles such as the vacuoles, Golgi apparatus, and endoplasmic reticulum (Lecourieux et al., 2002). Ca^{2+} spikes caused by elicitors mediate subsequent signaling events and also further amplify Ca^{2+} signaling (Price et al., 1994; Lecourieux et al., 2002).

Fungal elicitation triggers biphasic ROS generation usually within the first 10-30 minutes of contact with elicitor with a second phase occurring 1-3 hours later (Bolwell and Wojtaszek, 1997; Zhao et al., 2005). ROS further induces other plant defense responses including cross-linking structural protein and lignin polymers to strengthen the cell wall, and to induce hypersensitive cell death, defense gene activation, and defensive compound production (Levine et al., 1994). In certain plant species, general ROS is sufficient for the accumulation of secondary metabolites while in other H_2O_2 specifically mediates the production of these defensive compounds (Degousee et al., 1994; Guo et al., 1998).

There are several studies on how the presence of chitosan specifically affects the production of AN; these studies are summarized in Table 3. Lei et al., (2011) observed that foliar application of chitosan (0.1 mM) increased AN concentration by 53% when compared to untreated controls at 48 hours post-application. Test plants were grown in a greenhouse and all leaves were harvested, dried and powdered prior to extraction of AN that was quantified by high performance liquid chromatography (HPLC). The study concluded that AN concentration in *A. annua* increased after the foliar application of chitosan, which seems to have contributed to increased expression CYP71AV1 and increased ROS production.

Yin et al., (2012) also studied elicitation effects of chitosan in *A. annua*, but used a chitosan oligosaccharide (COS), and found there was no significant increase in AN concentration after 48 hours post-application of COS (1 mM) to the shoots. Plants were grown in a growth chamber and leaves were harvested, freeze-dried and homogenized to a powder. AN concentrations were quantified using liquid chromatography mass spectrometry (LCMS). The study concluded that elicitation may not be a universal method for increasing AN concentration in *A. annua.*

Table 3: Summary of prior CH and SA shoot elicitation of *A. annua*

Elicitor	**SA**			**Chitosan (COS)**	**Chitosan**
Site of application	Shoots				
Type of cultivation	Field Cultivation	Lab cultivation	Lab cultivation		Greenhouse cultivation
Conc. yielding highest AN conc.	1.00 mM	1.00mM	7.2 mM	1.00 mM	0.1 mM
Time yielding highest AN concentration	90 days	96 hours	48 hours	48+ hours	48 hours
Quantification	HPLC	HPLC	LC-MS		HPLC
Extraction	Dried at 80°C/ petroleum	Dried at 40-45°C/ petroleum	Freeze-dried/ dichloro-methane		Dried at 40°C/ petroleum
AN increase	Significant	Significant	Not Significant		Significant
Reference	Aftab et al., 2011	Pu et al., 2009	Yin et al., 2012		Lei et al., 2011

2.3.3 Salicylic acid induced elicitation

Salicylic acid (SA), a monohydroxybenzoic acid, (Figure 9; adapted from (Loake and Grant, 2007) is a recognized inducer of plant systemic acquired resistance (SAR) resulting from plant-pathogen contact. SAR is a resistance response of the whole-plant following a contained exposure to a pathogen. However, SA is not a universal inducer of the production of secondary metabolites that act as defensive components. During a pathogen attack and plant hypersensitive reaction, SA will quickly accumulate at the site of infection. It will spread throughout the plant further stimulating a range of plant defense responses. SA will stimulate

gene expression related to the biosynthesis of certain secondary metabolites (Taguchi et al., 2001).

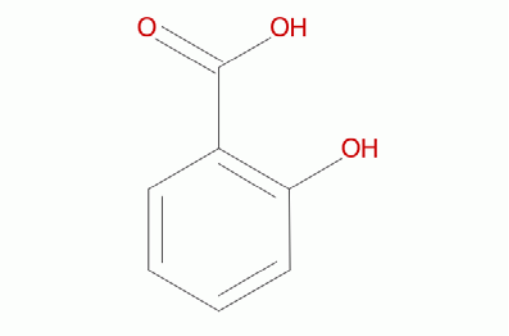


Figure 9: Salicylic structure

Several studies showed that SA elicits AN production in *A. annua* with varying levels of success (Table 3). One study by Pu et al. (2009) showed that an SA solution (1.0 mM) applied to the leaves of *A. annua* (Chinese 001 strain) doubled the concentration of AN at 96 hours when compared to the untreated controls at 96 hours. Plants were grown in a growth chamber, harvested by cutting 3 cm from the soil and drying at 40-45^{o}C before AN quantification with HPLC. The study showed that SA increased total AN and also concluded that SA increased CYP71AV1 transcriptional levels; they suggested that the increased transcription was responsible for stimulation of the AN biosynthetic pathway in *A. annua.*

The elicitation effect of SA was also examined by Aftab et al. (2011). Field cultivated *A. annua* plants were exposed to salt stress and then sprayed with various concentrations of SA every 10 days for 90 days. Plants were harvested with roots intact and dried at 80^{o}C. HPLC analysis determined AN content in the leaves was higher in SA-treated plants under salt tress. The study concluded that this increase in AN production may be explained by enhanced ROS production that is induced by SA.

Yin et al. (2012) also investigated the elicitation effect of SA. In contrast to the above studies, however, their results showed that foliar application of SA solution (7.2 mM) to the shoots of *A. annua* did not significantly increase AN concentration in the leaves. Plants were grown in a growth chamber and leaves were harvested, freeze-dried and homogenized to a powder. AN concentrations were quantified using LCMS. Their study concluded that elicitation may not be a reliable and universal method for increasing AN concentration in *A. annua*. Thus,

the effect of chitosan and salicylic acid on AN elicitation is not clear and this project intends to help clarify the response.

2.3.4 *A. annua* root-signaling stimulates AN production in the shoots

Ferreira and Janick (1996) showed that when *in vitro*-cultured *A. annua* shoots developed roots, shoots produced more AN that shoots that did not develop roots. Mannan et al. (2010) also investigate the role of roots in AN production and concluded that rooted-shoots growing in liquid culture had a higher AN concentration in response to DMSO elicitation when compared to the unrooted-shoot control. Similar results were concluded from a study by Nguyen et al. (2012), which showed that when roots were present AN precursors were low in shoots while AN levels were high. In contrast, when roots were removed from shoots, AN precursors were high and AN levels were low concluding that roots are need to drive the final reaction from dihydroartemisinic acid to AN (Nguyen et al., 2012). Despite producing no AN themselves, these studies suggested that roots play a role in AN production, and so our study will investigate root vs. shoot elicitation.

Chapter 3: Hypothesis and Objectives

Preceding this study, to our knowledge no one has compared root vs. shoot elicitation in *Artemisia annua*. Prior artemisinin (AN) work also suggested roots play a key role in AN production (Ferreira and Janick, 1996; Mannen et al., 2010; Nguyan et al., 2012), so this project will specifically examine what, if any role roots play in the elicitation of AN production in the leaves of *A. annua.* Our hypotheses were as follows:

1) Application of chitosan (CH) and/or salicylic acid (SA) via the shoots increases both AN and flavonoid production in shoots of *A. annua* L.
2) Application of CH and/or SA via the roots increases both AN and flavonoid production in shoots of *A. annua* L.

Our objectives were as follows:

1) To measure at 48 and 96 hours after CH application to shoots the concentration of AN and flavonoids in both the undeveloped shoot tip leaves (hereafter referred to as TIPs) of *A. annua* and the 3rd thru 5th (fully expanded) leaves from the tip.
2) To measure at 96 and 120 hours after SA application to shoots the concentration of AN and flavonoids in both the TIPs of *A. annua* and the 3rd thru 5th leaves from the tip.
3) To measure at 48 and 96 hours after CH application to roots the concentration of AN and flavonoids both the TIPs of *A. annua* and the 3rd thru 5th leaves from the tip after exposing roots to salicylic acid.
4) To measure at 96 and 120 hours after SA application to roots the concentration of AN and flavonoids in both the TIPs of *A. annua* and the 3rd thru 5th leaves from the tip.

Chapter 4: Methodology

4.1 *In vitro* growth of *A. annua* (SAM)

Shoots of *Artemisia annua* L., SAM cultivar, were grown *in vitro* in Magenta boxes containing 50 ml of rooting medium (pH 5.8) consisting of 5 g/L of agar gel (Phytotechnology Laboratories; Prod # A133; Agargellan), 20 g/L of sucrose (Phytotechnology Laboratories; Prod # S391) and 2.15 g/L of Murashige and Skoog (MS) medium with vitamins (Phytotechnology Laboratories; Prod # M519). Cultures were grown in continuous cool white fluorescent light at 25^{o}C at approximately 100 µmol m^{-2} sec $^{-1}$. Once significant roots were established (as determined by visual inspection, approximately three weeks), cultures were moved from their *in vitro* environment and planted in perlite (Perlite Vermiculite Packaging Industries Inc., OH; coarse grade) in individual 2x2 seedling boxes (5cm x 5 cm x 6cm). Plants were then placed in Pyrex dishes (5cm x 20cm x 30cm) with 25% Hoagland's solution (Hoagland and Arnon, 1950) at pH 5.8 (see Appendix A for recipe). Plants were grown in a growth chamber at 25^{o}C under continuous cool white fluorescent lights at approximately 40 µmol m^{-2} sec $^{-1}$.

4.2 Treatment with chitosan

Chitosan (CH) was purchased from Sigma-Aldrich Chemical Company (product number C-3646) and prepared according to the following protocol: CH was dissolved in 5% acetic acid, diluted in distilled water to 100mg/L, and pH adjusted to 6.5 with 2M NaOH. CH was applied to A. *annua* according to the method of Lei et al., (2011). All plants had at least 15 leaf nodes before experiments began. For shoot application, a minimum of eight plants was individually sprayed with 10 ml of CH solution using a spray atomizer. After 48 hours, the 3rd-5th leaves (3rd fully expanded leaf from shoot tip) and the undeveloped shoot tip leaves (hereafter referred to as TIPs) (see Figure 10) of half of the plants were harvested. After 96 hours, the remaining plants of were harvested as previously described. Plants treated with 10 mL distilled water adjusted to pH 6.5 with NaOH served as a control and were harvested as previously described at 48 and 96 hours.

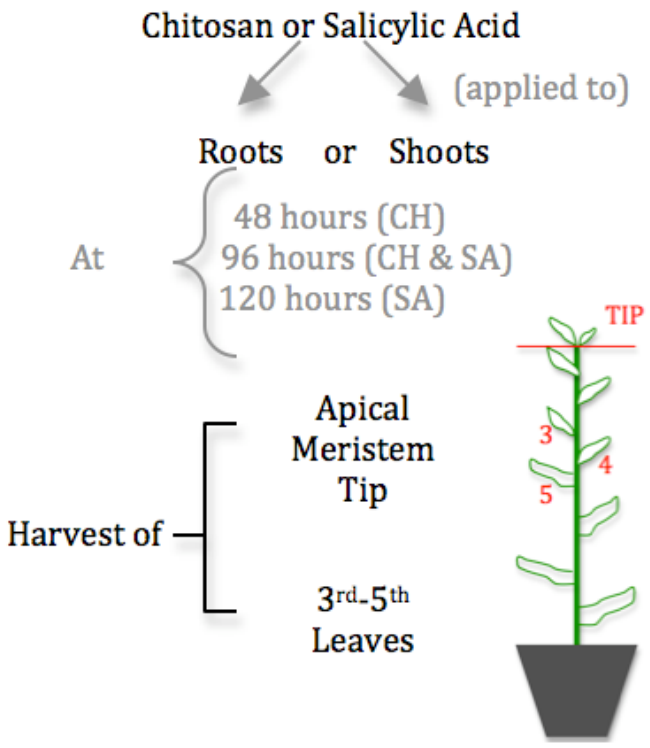


Figure 10: Elicitation experiment summary.

For root application, 10 ml of CH solution was individually applied to the top of the perlite medium surrounding the base of a minimum of eight plants. Care was taken to ensure each plant had the same exposure to compound and that leaves were not touched with the elicitor. After 48 hours, the 3rd-5th leaves and the shoot tips of half of the plants were harvested. After 96 hours, half of the plants were harvested as previously described. To create a control, 10 mL of distilled water at pH 6.5 were added to the top of the perlite medium of a minimum of four plants and were harvested as previously described at 48 and 96 hours.

4.3 Treatment with salicylic acid

Salicylic acid (SA) was purchased from Sigma-Aldrich Chemical Company (product number S-0875), and prepared and applied to *A. annua* according to the method of (Pu et al., 2009) using a 1 mM SA solution, pH adjusted to 6.5 with 2M NaOH. Before the experiment, plants were screened to ensure all samples had at least 15 leaf nodes. For shoot application, a minimum of eight plants was individually sprayed with 10 ml of the SA solution using a spray atomizer. The leaves were harvested as previously described at 96 and 120 hours. A minimum

of four plants treated with 10 mL distilled water adjusted to pH 6.5 with NaOH served as a control and were harvested as previously described at 96 and 120 hours.

For root application, 10 ml of SA solution was individually applied to the top of the perlite medium surrounding the base of a minimum of eight plants. Care was taken to ensure each plant had the same exposure to compound and that leaves were not touched with the elicitor. The leaves were harvested as previously described at 96 and 120 hours. To create a control group, 10 mL of distilled water at pH 6.5 were added to the top of the perlite medium of a minimum of four plants and were harvested as previously described at 96 and 120 hours.

4.4 Extraction

At each sample time, leaves 3-5 were pooled per plant and weighed. A single TIP per plant was weighed (FW) and extracted using methylene chloride (Fisher Scientific, product number D37-4). Samples placed in individual test tubes were sonicated in water at 25°C for 30 minutes to extract artemisinin (AN), deoxyAN as well as flavonoids. Solvent was decanted and concentrated by evaporation with nitrogen gas in an N-evap. Test tubes containing the dried extract were stored at -20°C.

4.5 Thin layer chromatography

Thin layer chromatography (TLC) was performed on two representative plates to provide a comparative profile of AN and flavonoids and to also determine the appropriate amount of each sample that was needed for Gas Chromatography-Mass Spectrometry (GCMS). An amount equal to 0.01 g FW of the pooled-leaf sample and an amount equal of 0.005 g FW of the TIPs were spotted on a silica gel plate. TLC was performed by using the solvent system: toluene-ethyl acetate, 2:1. Flavonoids were visible as a dark gray-purple spots under UV light. Casticin R_f was 0.29 and artementin R_f was 0.36. Plates were stained with an anisaldehyde spray consisting of glacial acetic acid, concentrated sulfuric acid, and *p*-anisaldehyde, 50:1:0.5, causing AN to appear pink, R_f =0.61 after heating at 110 °C for 10 minutes (Pras et al., 1991).

After spraying, the plate was heated at 110°C for 10 minutes, in a method developed by Pras et al., (1991). R_f values were calculated by the following equation:

$$R_f = \frac{\text{Migration distance traveled by substance}}{\text{Migration distance of solvent front}}$$

4.6 Gas-chromatography mass spectrometry

To quantify the amounts of AN in each sample, GCMS was used according to the protocol of Weathers and Towler et al. (2012): Extracted samples were suspended in methylene chloride (CH_2Cl_2) and 0.01 g of fresh weight was transferred to a 1 mL vial with a 100 µL insert. A 1 µL aliquot was injected into the GCMS [GC, Agilent 7890A; MS, Agilent 5975C; column, Agilent HP-5MS (30 m ×0.25 mm ×0.25 µm)] and AN separated using the following oven program: ion source temperature 280°C; inlet 250°C; initial temperature of 125°C for 1 min, then ramp to 300°C at 5°C/min, for a total time of 36 min. Ultrapure He was the carrier gas at 1 mL min^{-1}. Identification was via NIST library and AN standard (Sigma-Aldrich Chemical, St. Louis, MO).

4.7 $AlCl_3$ assay to quantify flavonoid concentrations

Total flavonoid concentration was measured using the aluminum chloride ($AlCl_3$) assay according to protocol of Meda et al. (2004). Quercetin was used to generate a standard curve by determining the optical density (OD) of various concentrations of quercetin at 415 nm. Quercetin was dissolved in methylene chloride and aliquots of 10, 20, 30, and 50 µg were placed in glass test tubes. Solvent was evaporated with nitrogen gas using an N-evap. Each sample was re-suspended in 1.5 mL of MeOH before 1.5 mL of the 2 % (w/v) $AlCl_3$ reagent was added to each tube to maintain a 1:1 ratio of MEOH to the $AlCl_3$ reagent. Samples were capped with a glass marble to prevent evaporation and incubated at room temperature for 25 minutes. A blank was prepare by adding 1.5 mL of MeOH and 1.5 mL of the $AlCl_3$ reagent to a glass test tube, capped, and incubated for 25 minutes at room temperature. After the incubation period, OD at 415 nm was measured using a Spec 20 spectrophotometer. Quercetin concentration (µ/mL) was plotted against OD to generate a standard curve and a linear equation. See Figure 11.

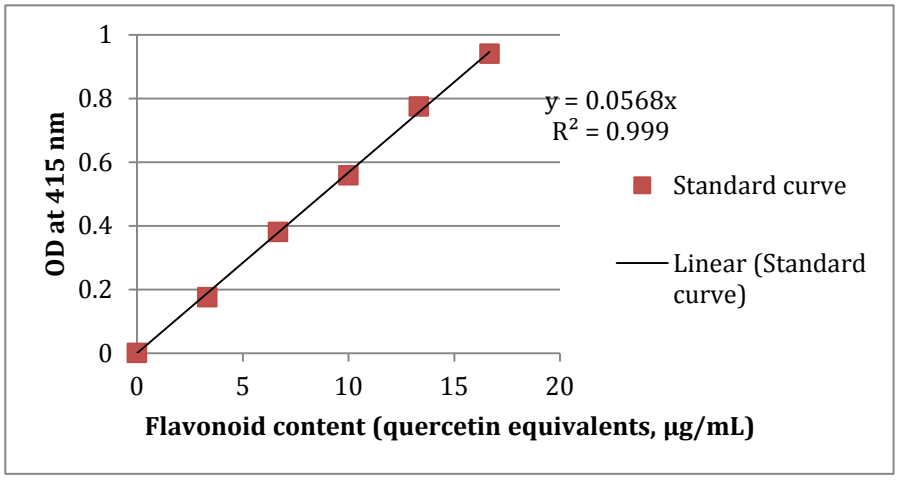


Figure 11: Quercetin standard curve for flavonoid concentration quantification using $AlCl_3$.

Dried extract samples of both leaf and TIP extract samples were re-suspended in methylene chloride and aliquots of a volume of extract equal to 0.005 g FW and 0.01 g FW of the respected samples were transferred to glass test tubes and dried. Samples were re-suspended in 1.5 mL MeOH and 1.5 mL of 2% (w/v) $AlCl_3$ reagent was added, capped, and incubated for 25 minutes before OD at 415 nm was measured.

4.8 Statistical analysis

All experiments were repeated twice and each experiment had a minimum of 2 samples. Results from each experimental group were pooled together and averages were calculated and compared for statistically significant results using independent t-tests.

5: Results

A. annua (SAM cultivar) was grown hydroponically and was treated with either chitosan (CH) or salicylic acid (SA) to the roots or shoots. After 48 and 96 hours (CH treated plants) and 96 and 120 hours (SA treated plants) the undeveloped shoot tip leaves (hereafter referred to as TIPs) and the 3rd-5th full expanded leaves from the tip of were harvested. Artemisinin (AN) and flavonoids were then extracted and profiled using thin layer chromatography (TLC) and quantified with gas chromatography mass spectrometry (GCMS) and $AlCl_3$ assay, respectively. Figure 10 (Chapter 3, page 24) shows a summary of the elicitation experiments.

5.1 Thin layer chromatography of CH and SA treated *A. annua* extract

Thin layer chromatography (TLC) was performed on two representative plates to determine the presence of secondary metabolites, specifically AN and flavonoids. Flavonoid presence indicated by deep purple spots on plates containing leaf extracts (Figure 14A) and TIP extracts (Figure 14B). After plates were sprayed with *p*- anisaldehyde, AN presence was shown by hot pink spots on the same plates containing leaf extract (Figure 14C) and TIP extract (Figure 14D). Each plate contained extracts of plants treated with either CH or SA and their respective controls at each time point and application site, roots vs. shoots. At least three flavonoids and three terpenoid separated and were visible under UV light and after *p*-anisaldehyde spray.

5.2 Chitosan root and shoot elicitation in *A. annua*

CH solution was applied to either the roots or shoots of *A. annua* and at 48 and 96 hours post-application, the TIPs, and the 3rd-5th leaves from the shoot tip were harvested and compared to unelicited controls.

In the leaf extract, there was no increase in AN (Figure 13A), or deoxy AN (Figure 13B). At 48 hours, however, AN decreased significantly compare to the control (Figure 13A). Flavonoids also showed no significant changes after CH elicitation (Figure 13C).

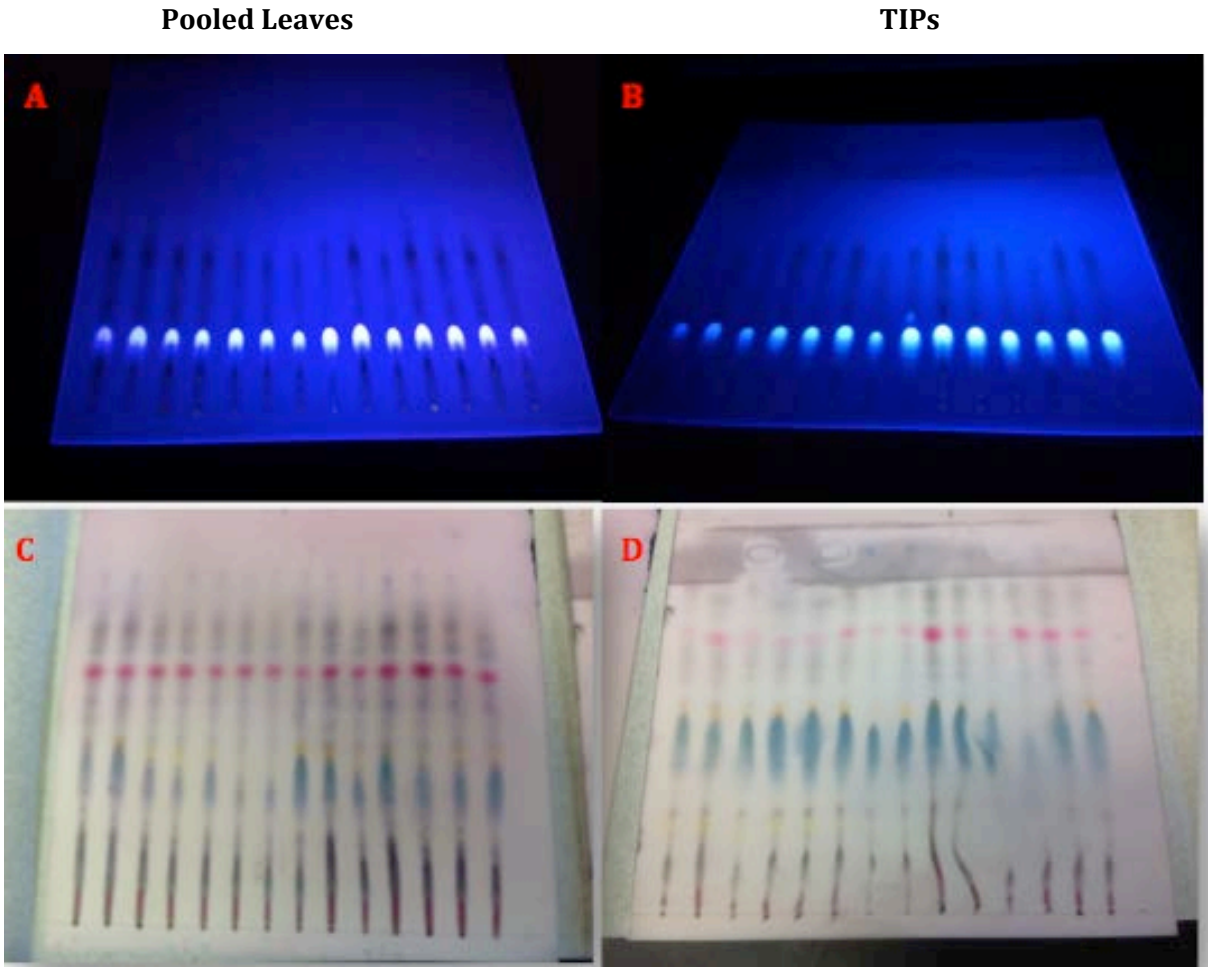


Figure 12: TLC representation plates.

TLC representation plates of leaf and TIP extracts under UV light (A&B) and visible light after *p*- anisaldehyde spray (C&D). Each plate had representation aliquots of extracts in the following order: CH48r, CON48r, CH48s, CON48s, CH96r, CH96s, CON96r, CON96s, SA96r, SA96s, SA120r, CON120r, SA120s, CON120s (CH=chitosan, SA= salicylic acid, CON= control, #=time harvested, r=root, s=shoot).

When AN, deoxyAN, and flavonoids were measured in the TIPs of the same CH treated plants, a different response emerged. AN increased at both 48 hours and 96 hours when compared to the control, but was only statistically significant at the earlier time point and only for root-elicited plants (Figure 14A). The level of flavonoids increased at both time points when compared to the control but was only significant at 96 hours and only in root-elicited

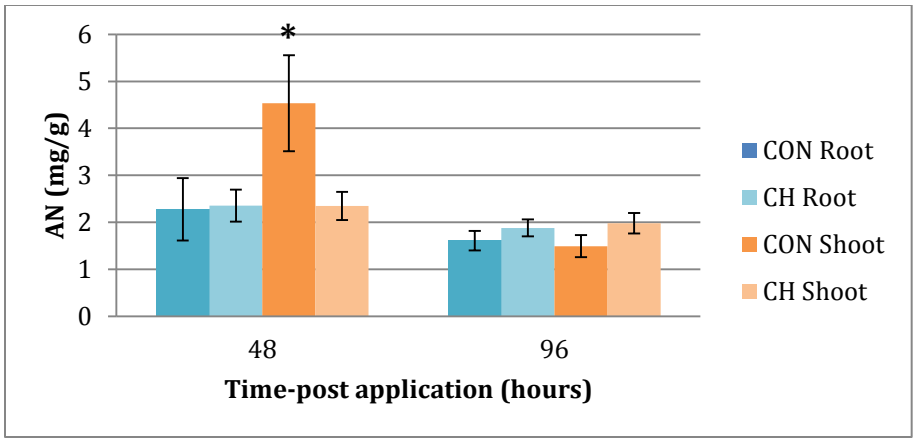
plants (Figure 14C). Although deoxyAN showed increases similar to AN, the data were not statistically significant (Figures 14B).

5.3 Salicylic acid root and shoot elicitation in *A. annua*

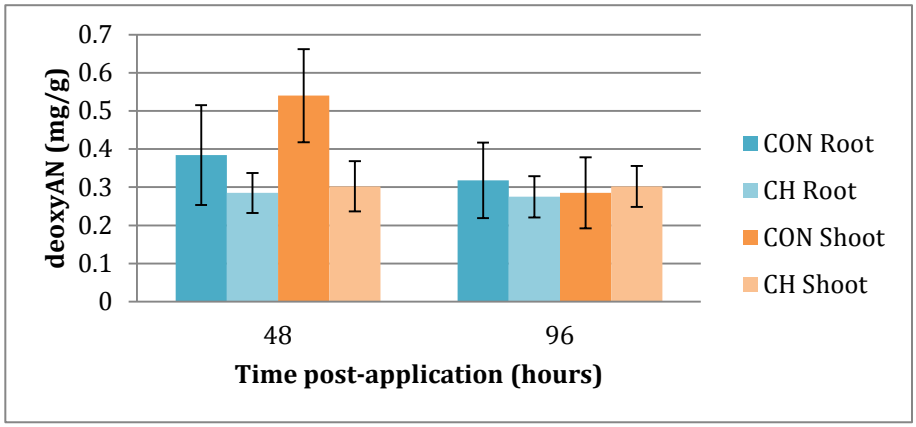
SA solution was applied to either the roots or shoots of *A. annua*. At 96 and 120 hours post-application, the TIPs and the 3rd-5th leaves from the apical meristem of each plant were harvested and compared to unelicited controls.

In the leaf extract, there was no increase in AN (Figure 15A), or deoxy AN (Figure 15B). Flavonoids also showed no significant changes after SA elicitation (Figure 15C).

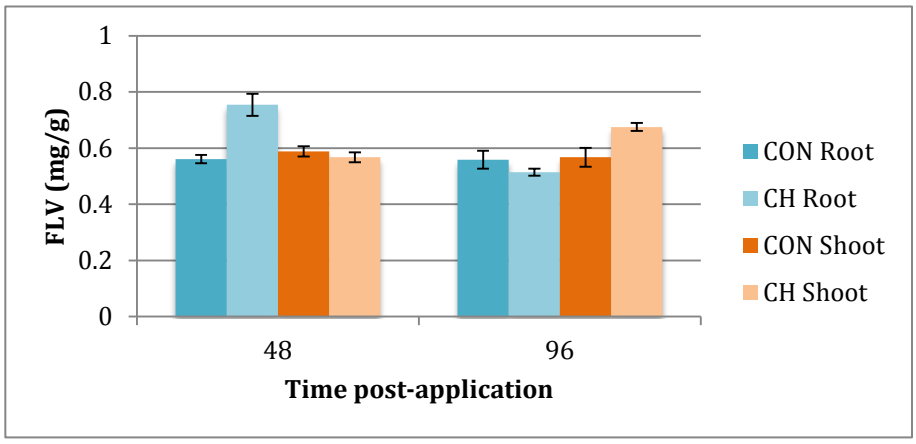
AN, deoxyAN, and flavonoids were measured in the TIPs of the same SA treated plants, a different response emerged. AN increased at both 96 and 120 hours, but only for root-elicited plants but was not statistically significant (Figure 16A). DeoxyAN showed similar increases and was statistically significant at 96 hours for root-elicited plants (Figure 16B). Flavonoids increased at both time points and both sites of application but data were only statistically significant at 96 hours post elicitation only in root-elicited plants (Figure 16C).



A

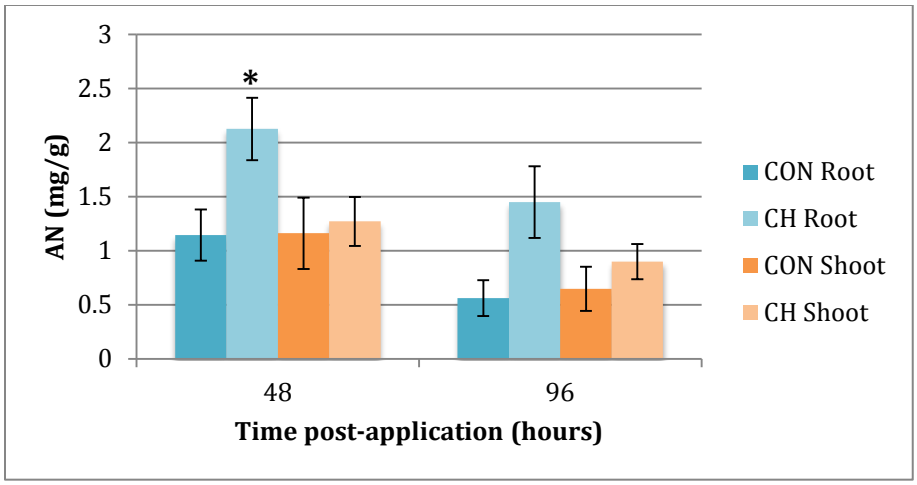


B

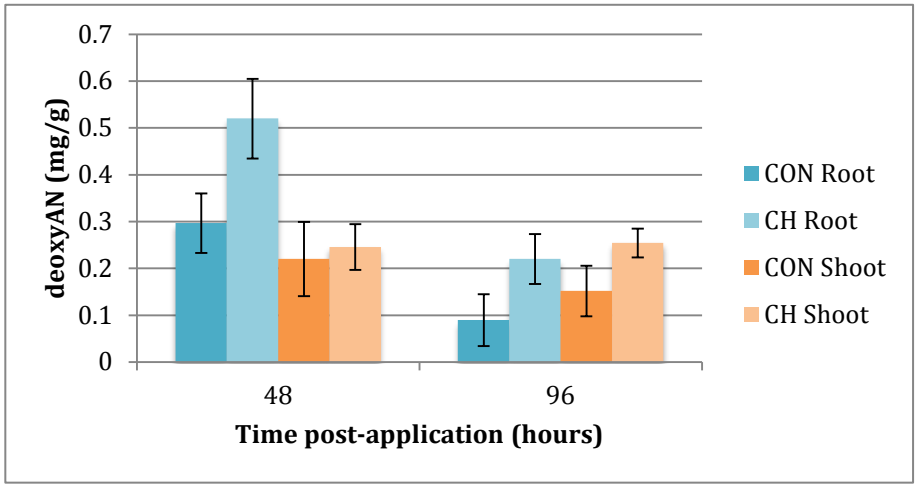


C

Figure 13: AN, deoxyAN, & FLV concentration in leaves after CH elicitation
FLV=flavonoids, bars show ±SE, n≥4, *significant at p≤0.05 vs. control.



A



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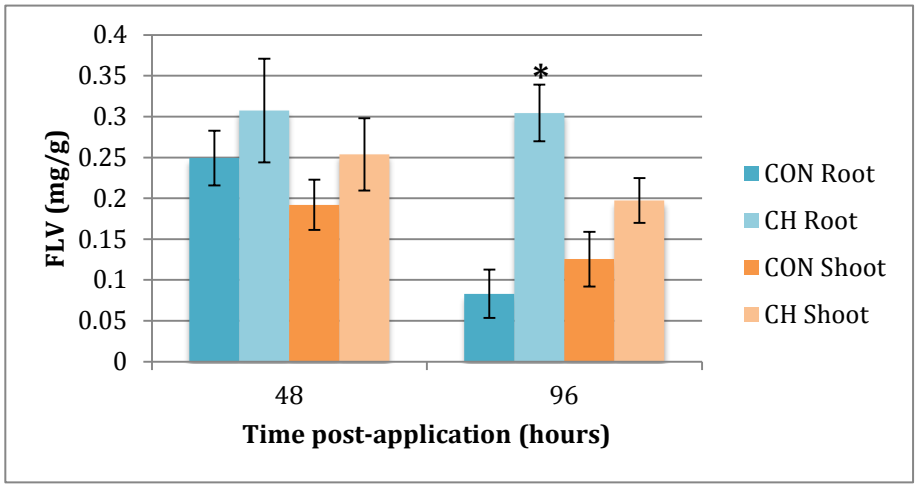
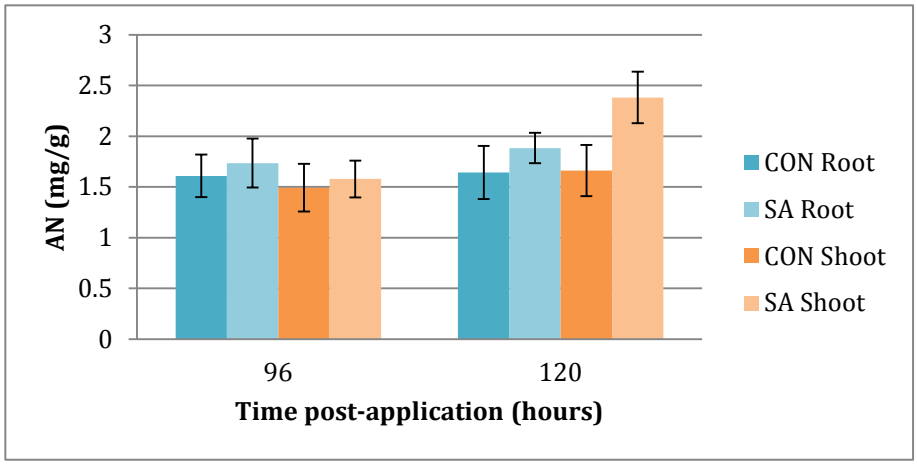
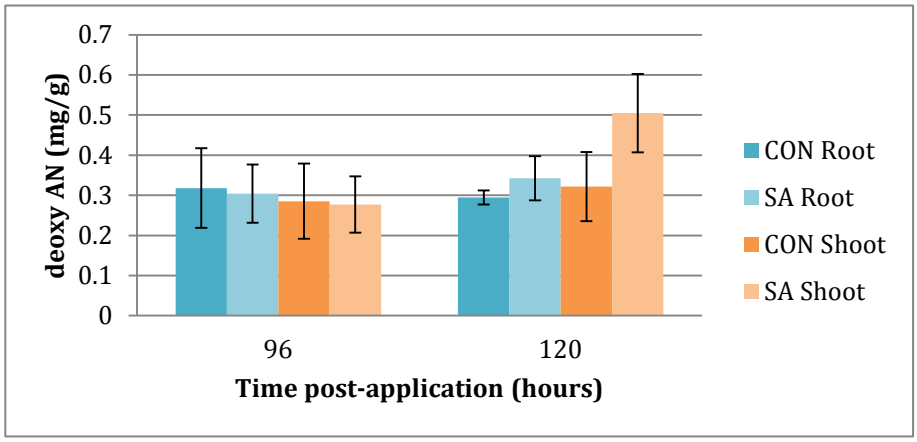


C

Figure 14: AN, deoxyAN, & FLV concentration in TIPs after CH elicitation.
FLV=flavonoids, bars show ±SE, n≥4, *significant at p≤0.05 vs. control.



A



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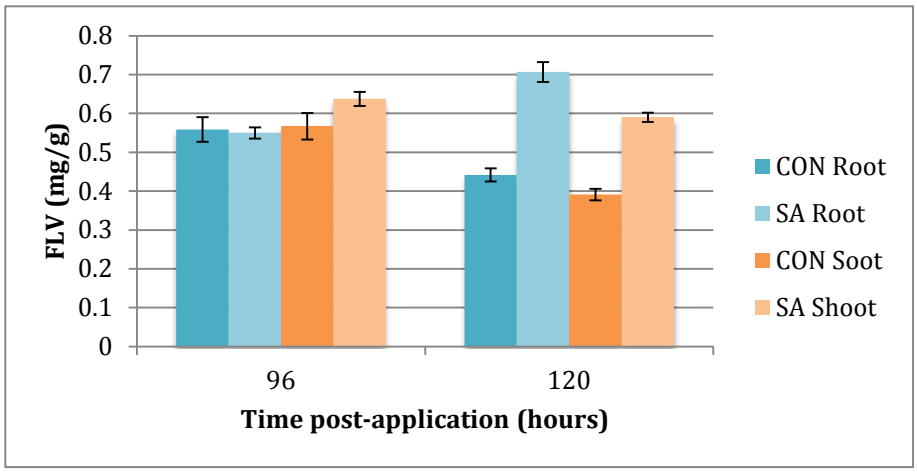
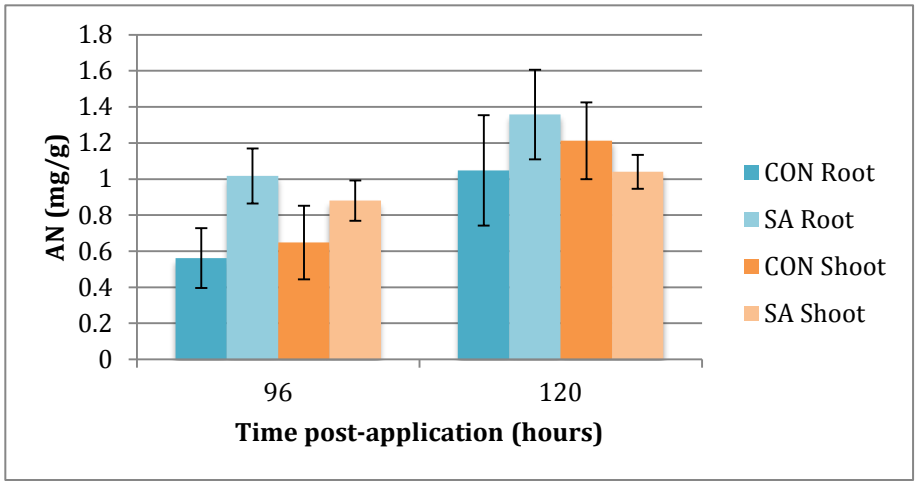
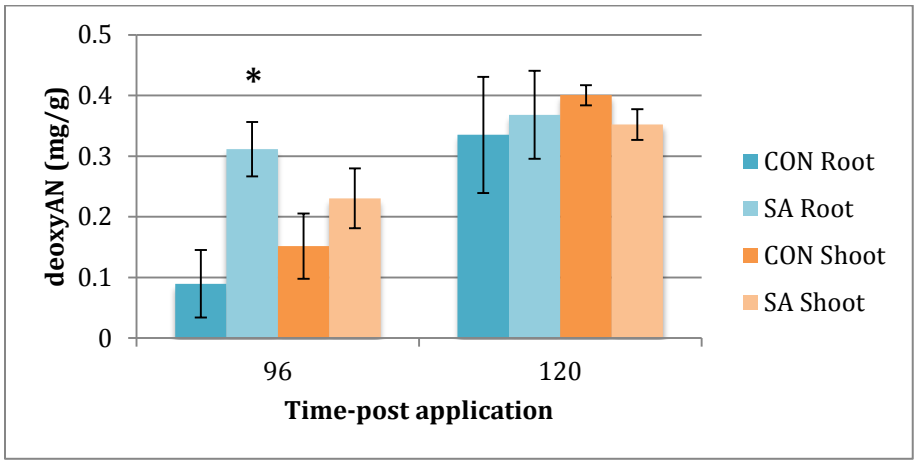


C

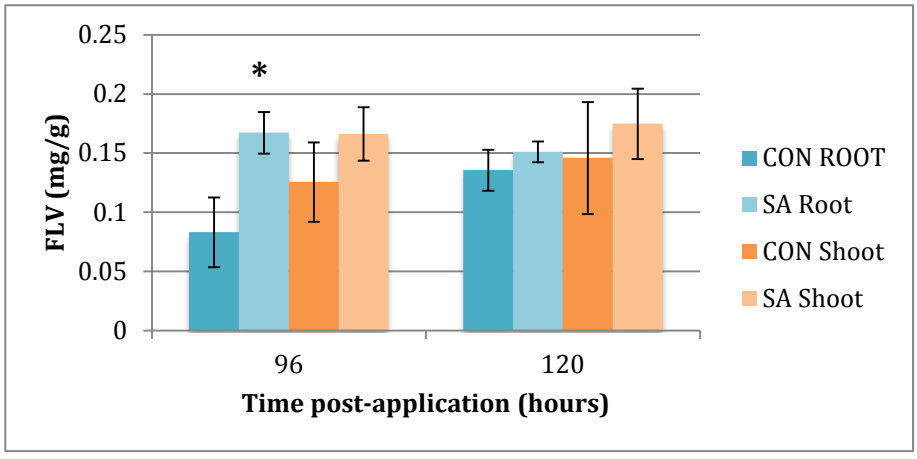
Figure 15: AN, deoxyAN, & FLV concentration in leaves after SA elicitation.
FLV=flavonoids, bars show ±SE, n≥4, *significant at p≤0.05 vs. control.



A



B



C

Figure 16: AN, deoxyAN, & FLV concentration in TIPs after SA elicitation
FLV=flavonoids, bars show ±SE, n≥4, *significant at p≤0.05 vs. control.

6: Discussion

When SA or CH was applied to either the roots or shoots of *A. annua*, there was no significant increase in AN, deoxyAN, and flavonoids in the mature leaves of *A. annua* compared to the appropriate respective control. In contrast, when the undeveloped shoot tip leaves (hereafter referred to as TIPs) were examined, a clear trend emerged in elicited plants, showing increases in AN, deoxyAN, and flavonoid levels from both CH and SA compared to their corresponding controls. Moreover, significantly higher levels of AN were measured in elicited plants than in the control after 48 hours of CH elicitation via the roots. Indeed at 48 hours in particular, CH elicitation via the roots yielded significantly higher levels of AN than the corresponding control. Although no other significant increases occurred, the CH-treated plants generally produced more AN, deoxyAN, or flavonoids than their corresponding controls at both 48 and 96 hours via both root and shoot elicitation.

Similar responses occurred in plants treated with SA. The TIPs produced more secondary metabolites than controls, but only when elicited via the roots. The mature leaves, on the other hand, showed no increases.

The results of this study did not completely correspond to the small number of previous studies of CH elicitation of *A. annua*. Lei et al. (2011) showed that foliar application of CH at 0.1 mM significantly increased AN content in *A. annua*. Our study however, did not yield such increases. In contrast, when Yin et al. (2012) applied a 1 mM CH solution to the shoots of *A. annua*, there was a slight increase in AN in the leaves, but results were not statistically different from the control. It is important to note, that in our study, AN extraction was done on fresh leaves to determine the real time concentration of AN and flavonoids at 48 and 96 hours post elicitation. Lei et al. (2011), however, used heat-dried plant material for extraction, so it is possible that heating altered the concentration of AN in the leaves, and this may explain why Lei et al. (2011) observed significant increases in AN. On the other hand, Yin et al. (2012) used freeze-dried plant material, a method that should yield more real time results, and indeed their results showed that although AN levels increased in plants after CH treatment the increases were not statistically significant, a result consistent with our data.

The data of this study also do not completely correspond with other SA elicitation studies done by Pu et al. (2009) and Aftab et al. (2011). Both studies showed that 1 mM of SA solution applied to the shoots of *A. annua* significantly increased AN in the leaves. Our study,

however, did not yield such increases. Interestingly, a study by Yin et al. (2012) showed results similar to ours. They used a foliar spray of 7.2 mM SA and found no significant increases in AN in the shoots. As we already noted, our study extracted AN from fresh leaves in order to provide a real time AN measurement in the leaves at 96 and 120 hours post-application of SA. In contrast, Pu et al. (2009) and Aftab et al. (2011) heat-dried the plant material prior to extraction, so again heat may have altered the AN content. On the other hand, Yin et al. (2012) used freeze-dried plant material, and as already discussed, results were similar to ours. Overall this suggests that the post-harvest treatment of the plant material prior to extraction is critical.

Because TIPs but not leaves showed increases (some significant) in the amount of measured secondary metabolites, our data suggest that when SA or CH are applied to the roots, the juvenile leaves are more responsive to these elicitors than are the mature leaves. These results further substantiate the importance of the roots in the production of secondary metabolites in the juvenile leaves of *A. annua*.

To our knowledge, no studies have been done using *A. annua* to examine how flavonoid production is affected by either CH or SA elicitation. Other than the reports by Kapoor et al (2007) and Rapparini et al., (2008) that showed *Glomus* sp. induced AN and terpenoid production after mycorrhizal infection of *A. annua* roots, we are not aware of any studies on production of secondary metabolites in plants after elicitation via the roots. The results, therefore, of the flavonoid production and root elicitation provide new insight into the role of roots in elicitation responses in plants.

7: Conclusion

Artemisinin (AN) production is significant for its application in anti-malarial medications. We investigated increasing production of AN and its synergistic flavonoids through elicitation by applying chitosan and salicylic acid to the roots and shoots of *Artemisia annua.* This study showed that when chitosan was applied to the roots or shoots, increased levels of secondary metabolites were observed in undeveloped juvenile leaves, but not in mature leaves. Salicylic acid also increased secondary metabolites, but only in the juvenile leaves and only when applied via the roots. Although the roots of *A. annua* produce no AN, the results of this study are consistent with prior work showing that the roots play an important role in the production of artemisinin and other secondary metabolites especially in juvenile leaves.

8: Future Work

Results of experiments could have been improved with the addition of more plant samples for testing. The n value was lower than would have been preferred thus resulting in few statistically significant differences between controls and experimental plants. It is possible, for example, that if we had increased the n by performing additional chitosan (CH) and salicylic acid (SA) experiments had been performed, we would have increased the n value and, we may have observed more statistically significant results, especially in juvenile leaves when elicited via the roots.

While completing these experiments, we noticed certain techniques that could have been altered to potentially improve our result. When spraying the shoots with elicitors, the volume of solution was too great and leaves were excessively wetted. Droplets of solution may have, therefore, dripped off the plant and into the Hoagland nutrient solution below, whereby it could have been absorbed through the roots, which would have confounded our results. In future experiments, greater precaution is needed to cover the base of the plant to prevent the nutrient solution from becoming cross contaminated with shoot-applied elicitor. It is also suggested that instead of using a set spray volume, to instead stop the spray application after leaves are fully coated with spray and prior to the solution beginning to drip off the plant leaves.

When inoculating the roots with elicitor, initially the CH or SA solution was added to the base of the plant to the perlite that surrounds the roots. However, the amount of nutrient solution that the seedling boxes were partially submerged in was not taken into account. To insure the exposure of the roots of each plant to an equal amount (10 mL) of elicitor, it is suggested that seedling boxes be temporarily removed from nutrient solution, allowing excess solution to drain from the perlite (15 minutes) before the elicitor is added to the perlite. This would then allow for better absorption of the elicitor and hopefully provide more definitive (statistically significant) results.

Future experiments also could include additional elicitation treatments. We only inoculated the plants once and then waited for a specific time before harvesting. It is possible that with additional elicitation treatments, the leaves of *A. annua* would be more responsive to the elicitor. Another experiment could investigate the AN levels in juvenile leaves as they matured. If the time between inoculation and harvesting was extended, undeveloped shoot tips

leaves, could be allowed to develop in order to determine if the AN levels remains high, or if the AN decreases back to the levels found in the mature leaves.

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Appendix A:

Hoagland's Nutrient Solution (Hoagland and Arnon 1950)

Stock solutions:

1) 1M KNO_3 (101.1 g/l dH_2O)
2) 1M $Ca(NO_3)_2 \cdot 4H_20$ (236.09 g/l dH_2O)
3) 1M KH_2PO_4 (136.08 g/l dH_2O)
4) 1M $MgSO_4$ (120.37 g/l dH_2O)
5) 1M EDTA (37.2 g/l dH_2O)
6) 1 M $FeSO_4$ (151.53g/l dH_2O)
7) Other salts (add the following per 1 liter dH_2O)
    a. 2.8g H_3BO_3
    b. 1.9g $MnCl_2 \cdot 4H_20$
    c. 0.22g $ZnSO_4 \cdot 7H_20$
    d. 0.08g $CuSO_4 \cdot 5H_20$
    e. 0.027g $Na_2MoO_4 \cdot 2H_20$

To prepare 1 liter of 25% Hoagland's solution*

1. Measure 975ml of dH_2O
2. Add stock solutions in the following amounts
   5 ml KNO_3 solution (#1)
   5 ml $Ca(NO_3)_2 \cdot 4H_20$ solution (#2)
   2 ml $MgSO_4$ solution (#4)
   1 ml KH_2PO_4 solution (#3)
   1 ml EDTA solution (# 5)
   10 ml $FeSO_4$ solution (#6)
   1 ml "other salts" solution (#7)

   *Adjust pH to 5.8