Effects on *Artemisia annua* Growth with the Addition of Gibberellic Acid, Salicylic Acid, and Methyl Jasmonate in Hydroponic Systems

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ABSTRACT

Malaria is a global health issue that will continue to spread with the increase in mosquito populations caused by the rise in global temperatures. The most effective drugs currently available for malaria treatment contain the anti-malarial compound, artemisinin, derived from *Artemisia annua*. To potentially increase efficiency of artemisinin production in *A. annua*, I investigated the effects of adding gibberellic acid, salicylic acid, and methyl jasmonate in hydroponics on the growth of intact plants. The goal of the study was to determine the optimal concentrations of each elicitor that maximizes the growth of *A. annua*, investigate the effects of different combinations of these elicitors on growth, and to determine the optimal exposure period of each elicitor that maximizes growth. I hypothesized that the concentrations of these chemicals that increased growth in previous studies would also yield the best results in hydroponic systems. Also, I hypothesized that the combination of all of these chemicals would yield maximum growth by inducing growth through different pathways. In terms of the optimal exposure periods, I hypothesized that the rate of growth would decrease over time and stabilize at around four weeks. My results show that the treatments containing gibberellic acid demonstrated the highest dry weights, suggesting that gibberellic acid enhances *A. annua* growth. In addition, the combination treatment with all three elicitors yielded the lowest dry weights. The data shows that the optimal exposure period for all elicitors and combinations was between 8 weeks and higher, except for individual salicylic acid treatments that showed the greatest growth between weeks 4 and 6. Future studies can expand on this study by investigating the relationship between *A. annua* growth and the addition of gibberellic acid.

KEYWORDS

medicinal plants, chemical elicitors, malaria, artemisinin, plant growth

INTRODUCTION

Between 300 and 500 million people are currently infected with malaria, with more than two billion at risk for infection. Experts project a continued increase in these numbers. Two million people die annually from malaria, including one million children (Davies et al. 2009). The multidrug-resistant malaria parasite, *Plasmodium falciparum*, is found in many tropical countries and is particularly difficult to treat. The spread of malaria is dependent on the propagation of mosquitoes that transmit the disease by carrying the *Plasmodium* parasites. With the estimated global temperature increases (Houghton 2005, Wagner et al. 2009), mosquito populations will increase dramatically because warmer temperatures will allow them to migrate and propagate in new areas further north (Robert et al. 2002). In addition, cycles of heat and drought followed by heavy rains will occur more frequently, which will create more bodies of water that mosquitoes can use to reproduce (Weathers et al. 1994). The increase in vectors of transmission for malaria will create devastating health crises in tropical countries, specifically in Latin America and Sub-Saharan Africa (Robert et al. 2002). Global temperature projections suggest increases in both malaria transmission and distribution, stimulating investigations on methods to decrease costs and increase efficacy of malaria treatment.

Malaria treatment generally includes the use of the anti-malarial compound, artemisinin, derived from the plant *Artemisia annua* L. (Asteraceae), also called qinghao or sweet wormwood (Ferreira 2007, Greenwood et al. 2008). The World Health Organization currently recommends the use of artemisinin-based combination therapies (ACTs) in tropical regions where the malarial parasite has developed resistance to traditional anti-malarial drugs (Mutabingwa 2005). ACTs are the best drugs currently available for malaria treatment (Mutabingwa 2005), and drugs that contain artemisinin derivatives have almost 100% cure rates with usually only a three-day regimen (Congpuong et al. 2010). In addition to killing the *Plasmodium* parasite, artemisinin derivatives lower the rate at which resistance develops by reducing the survival of parasitic gametocytes. Reducing the number of viable *Plasmodium* gametocytes leads to fewer infectious germ cells, leading to decreased infectivity of mosquitoes and reduced parasite reproduction in the mosquito (Adjuik et al. 2004). Artemisinin derivatives kill all stages of the malaria parasite by interacting with heme in the blood and damaging the parasitic cells' microorganelles and membranes (Balint 2001). Some examples of artemisinin-derived molecules are artesunate, arteether, artemether, and dihydro-artemisinin (DHA) (Baldi 2007). There are currently no

known cases of parasitic resistance to artemisinin-based compounds, and resistance has not yet been successfully induced in a laboratory setting (Mutabingwa 2005). Artemisinin compounds have a particularly rapid clearance time (the rate at which a substance is removed from the blood), making them suitable for treating early cases of malaria to avoid progressions toward severe cases that lead to mortality in developing countries (Mutabingwa 2005). Therefore, increasing efficiency in artemisinin production to reduce costs of artemisinin-based medications is an important step in addressing the global spread of malaria. Increased supplies and reduced costs will facilitate increased use of ACTs and reduce rates of infection and mortality as well as infectivity of the vectors of transmission (Adjuik et al. 2004).

Enhancing the production of artemisinin in *A. annua* has been a goal for many research groups. The low yield of artemisinin within the *A. annua* plant is a serious constraint on the mass production of artemisinin-based drugs (Abdin et al. 2003, Weathers 1994). The average concentration of artemisinin in *A. annua* is 0.01-0.8% of the plant mass, which is very low compared to other compounds of this type (Van Agtmael et al. 1999). In addition to extraction from plant tissues, artemisinin can also be chemically synthesized; however, its production is complicated and unfavorable due to very poor yields and extremely high costs (Mutabingwa 2005, Ravindranathan et al. 1990). Therefore, increasing yield in intact plants is a more cost-effective production method compared to commercial synthesis. In the intact *A. annua* plant, artemisinin is synthesized from the mevalonate-terpenoid pathway in the glandular trichomes of the leaves, and artemisinin levels are highest in the shoots during the flowering stage (Shukla et al. 1991). One study investigating the distribution of artemisinin in five-week old *A. annua* plants found that the highest artemisinin content was found in the leaves of the upper parts of the plant (Jaziri 1992).

Adjustments in chemical elicitors have been used as an effective method to increase both *A. annua* growth and artemisinin yield. Gao-Bin et al. (2009) found that feeding elicitors in *A. annua* plant cell cultures enhanced plant growth and production of secondary metabolites, including artemisinin. Specifically, three elicitors, gibberellic acid, salicylic acid, and methyl jasmonate have been shown to stimulate growth and artemisinin production in hairy root cultures (Smith et al. 1997, Baldi and Dixit 2007, Gao-Bin et al. 2009). Smith (1996) examined the effect of six different concentrations of gibberellic acid in *A. annua* plant culture and found that the 0.01 mg/L concentration produced the highest yield of artemisinin and increase in growth. Gao-

Bin et al. (2009) provide evidence that salicylic acid (SA) can activate *A. annua* growth and artemisinin production. At 96 hours after SA (1.0 mM) treatment, the concentration of artemisinin was 54 % higher than that of control plants (Gao-Bin et al. 2009). Baldi and Dixit found that the addition of methyl jasmonate as an elicitor at 5 mg/L led to increases in both biomass and artemisinin content (Baldi and Dixit 2007). Although these previous studies successfully induced increases in *A. annua* growth and artemisinin production in tissue culture, Wang and Tan (2002) found that artemisinin content in intact plants of *A. annua* are much higher than in tissue culture. Therefore, determining optimal concentrations of the above elicitors to increase plant growth and artemisinin production in intact plants of *A. annua* is essential.

. Past studies have shown the correlation between increase in biomass and increase in artemisinin content as well as the localization of artemisinin in the foliar tissues of *A. annua*. This suggests that increases in biomass correspond to increases in artemisinin yields (Duke et al. 1994, Smith et al. 1997, Wang et al. 2002, Wang and Tan 2002). Therefore, increasing growth rates of *A. annua* is one method of enhancing artemisinin production efficiency.

Growing *A. annua* in hydroponic systems introduces a potentially cost-effective method for increasing artemisinin yields. The major advantages of hydroponic production include the large size and productivity of a hydroponic facility as well as the possibility for mass commercial production of plants in these facilities (Jones 2005). Hydroponics is the method of growing plants in a medium, other than soil, providing essential plant nutrient elements dissolved in water (Jensen 1999). A solution culture system, to be used in this study, is one type of hydroponic system that uses nutrient solution alone, with root systems completely immersed in solution (Jensen 1999). Variables such as light, temperature, humidity, and the composition of air can be easily controlled with hydroponics, maximizing overall biomass production (Resh 2004). The use of hydroponics minimizes the loss of root material, excess manipulation, and algae formation. In addition, the liquid nutrient solution that serves as the media for hydroponics provides more stable rooting conditions for the plant than in soil and prevents plant diseases caused by soil pathogens (Resh 2004). Although soil-free systems are not necessarily sterile, they contain significantly lower levels of microorganisms than soil systems. In hydroponic systems, all essential nutrients are readily and consistently available to the plant through the roots. Therefore, lower concentrations of nutrients are required, the pH of the nutrient solution can be maintained for optimal nutrient uptake, and there is no loss of nutrients from leaching (Jones 2005).

In my study, I investigated the effects of adding three elicitors in hydroponics on the growth of *A. annua* to determine if these elicitors have an effect on growth compared to the control, and to determine the optimal concentrations of each elicitor that increases plant growth. I hypothesized that all three elicitors would increase growth compared to the control and that the concentrations of each chemical that yielded best results in tissue culture would also yield the best results in hydroponic systems. I introduced varying concentrations of gibberellic acid, salicylic acid, and methyl jasmonate as elicitors in hydroponic systems to analyze changes in *A. annua* growth. Although past studies (Smith et al. 1997, Baldi and Dixit 2007, Gao-Bin et al. 2009) have conducted experiments with these chemicals as elicitors in tissue cultures, no previous studies have studied their effects in hydroponic systems. In addition to the effect of each elicitor, I investigated the effects of different combinations of these elicitors on *A. annua* growth. Each of these elicitors is involved in different chemical pathways within the plant; therefore, I hypothesized that combinations of these elicitors would yield greater increases in growth than each chemical separately. The alternative hypothesis was that these chemicals would have no effect on artemisinin yield. I harvested the plants at two week intervals to measure the effects of the elicitors over specific periods of time. I hypothesized that the growth rates would be high in the beginning and then would start to decrease. The alternative hypothesis was that *A. annua* growth rates remain constant with no changes over time. The costs associated with these methods are explored in the discussion. This study controlled for confounding factors including the amount of sunlight, pH, temperature, and insects affecting the plants.

METHODS

This study was conducted in collaboration with the Norman Terry Lab at the University of California, Berkeley, working with the State Key Laboratory of Biochemical Engineering in Beijing, China with Dr. Chun-Zhao Liu. I conducted the experiment at the South Greenhouse of the Oxford green houses at UC Berkeley in Berkeley, California.

Experiment Setup

A picture of the hydroponic setup

The *A. annua* seeds were germinated in pots starting September 11th 2009, and they were transplanted to hydroponic tanks for acclamation on November 11th 2009. The tank holds 40 liters of hydroponic solution and has 12 holes on the cover for the shoots to stay above the solution and the roots submerged. I selected plants that were similar in size (25–30 cm in height) to be transferred to hydroponic jars for the experiment. The plants were then transplanted into individual hydroponic jars on December 1st 2009. The hydroponic setup consisted of a 1-liter mason jar covered with aluminum foil and filled with hydroponic solution. The aluminum foil prevents sunlight from entering the jar, eliminating algae growth. I placed the germinated plant at the opening of the jar with a cap that holds the plant in place with the roots immersed in the hydroponic solution. I inserted a tube into the solution through a hole in the cap to provide a constant flow of oxygen for the plant. This study used one-tenth Hoagland's Plant Nutrient Solution (1938) containing the following macronutrients: 2 M KNO_3, 1 M KH_2PO_4, 2 M $MgSO_4$, 2 M $Ca(NO_3)$, 1 M NH_4NO_3, 0.4 M FeEDTA and the following micronutrients: 2.8 g/L H_3BO_3, 0.1 g/L $CuSO_4$, 1.8g/L $MnCl_2$, 0.2 g/L $ZnSO_4$, 0.025g/L Na_2MoO_4 (All chemicals ordered from Sigma Aldrich Company) . I monitored the transferred plants every other day for survival and significant observable changes, and changed the hydroponic solution every week to provide fresh nutrients and accurate elicitor concentrations for the plant.

I controlled for the potential confounding factors of light, pH, and temperature. To eliminate light as a confounding factor, I randomized the arrangement of plants on the table to account for possible differences in of light on different ends of the table. Preventing flowering was critical because flowering would cause them to stop growing. Because *A. annua* are short-day flowering plants, I used artificial light in the greenhouse from 4pm to 10pm to stop them from flowering as the days got shorter. I controlled the temperature in the greenhouse (South Greenhouse at UC Berkeley) at 27°C. I measured the pH during every solution change and maintained it at 7.3 – 7.4 for all treatments and controls. If the pH of the hydroponic solution was too high or too low, I adjusted the pH using hydrochloric acid (HCl) or potassium hydroxide (KOH), accordingly.

Addition of Elicitors

To determine the optimal concentration of gibberellic acid, salicylic acid, and methyl jasmonate that would increase the growth of *A. annua*, I added varying concentrations of these chemicals into the hydroponic solution. I used the concentrations that were determined to be most effective in previous studies as well as $1/5^{th}$ and 5 times these concentrations (Table 1). I tested over a range of concentrations for each elicitor.

Table 1. Treatment concentrations and replicates. Note the concentrations are based on previous studies performed on tissue cultures that increased *A. annua* growth and artemisinin yield.

Treatment	Concentration	Replicates
Control	No chemical treatment	5
Salicylic Acid 1 (SA 1)	200 μM	5
Salicylic Acid 2 (SA 2)	1 mM	5
Salicylic Acid 3 (SA 3)	5 mM	0*
Gibberellic Acid 1 (GA 1)	0.02 mg/L	5
Gibberellic Acid 2 (GA 2)	0.1 mg/L	5
Gibberellic Acid 3 (GA 3)	0.5 mg/L	5
Methyl Jasmonate 1 (MJ 1)	1 mg/L	5
Methyl Jasmonate 2 (MJ 2)	5 mg/L	5
Methyl Jasmonate 3 (MJ 3)	25 mg/L	5

* All plants died within the first two days.

To test the effect of adding different combinations of the chemical elicitors to the hydroponic systems, I tested five replicates for each combination, using the middle concentrations for each elicitor (Table 2).

Table 2. Elicitor combinations and replicates. Note the middle concentrations from Table 1 were used for each chemical.

Treatment Combination	Concentration	Replicates
SA + GA	1 mM + 0.1mg/L	5
SA + MJ	1mM + 5 mg/L	5
GA + MJ	0.1 mg/L + 5 mg/L	5
SA + GA + MJ	1mM + 0.1 mg/L + 5 mg/L	5

Exposure Period Experiment

To determine the optimal exposure period for elicitors on *A. annua*, one of the five different replicates for each treatment was harvested every two weeks. The harvesting dates were on December 15^{th} and 29^{th} 2009, January 12^{th}, 26^{th} 2010, and February 9^{th} 2010. I separated the

shoots and roots for drying purposes, oven-dried each sample in the drying oven, and measured the dry weight for each sample (Appendix A).

To determine the concentration of gibberellic acid, salicylic acid, and methyl jasmonate that yielded the best *A. annua* growth rates, I graphed the total mass versus the treatments. I calculated the slopes between each harvest to compare the growth rates for the different treatments and intervals. To compare the effects of individual elicitors and the combinations of elicitors, I graphed the total masses of those samples versus the treatments. I calculated the slopes for each interval to detect the combination that yielded the best growth rates. To determine the optimal exposure periods for each elicitor, the slopes for each harvest interval were compared to examine which intervals had the largest slopes for the different treatments.

RESULTS

Treating *Artemisia annua* in hydroponics with varying concentrations of gibberellic acid, salicylic acid, and methyl jasmonate showed that the addition of these elicitors have significant effects on plant growth rates. I measured growth by total mass recorded after each harvest. All of the elicitors, except for the SA/GA/MJ combination show increased growth rates compared to the control (Fig 1).

For the gibberellic acid treatment, all three concentrations (Fig 1b) showed a general trend of having higher growth rates than the control. Also, the average slopes for all three concentrations were higher than that of the control (Table 3). The GA 1 concentration showed the highest growth.

Both of the salicylic acid treatments show a greater growth rate compared to the control (Fig 1a), with the SA 1 treatment consistently having higher biomass than the SA 2 and control. The SA 3 plants treated with 5mM salicylic acid did not survive for the first solution change. In addition, both SA treatments had higher average slopes than the control (Table 3). The SA 1 concentration showed the greatest growth rates.

The plants treated with methyl jasmonate did not grow as fast as the gibberellic acid and salicylic acid treated plants (Fig 1c); however, the slopes between harvests for the MJ1 treatment with 1mg/L methyl jasmonate are consistently higher than for the control. The MJ2 and MJ3 treatments showed almost no increase in growth compared to the control (Table 3).

The combinations of elicitors, which were hypothesized to increase root and shoot growth, showed the greatest *A. annua* growth compared to control. The SA/MJ combination showed the greatest growth compared to the control consistently for all harvests. The GA/MJ and SA/GA combinations also showed greater growth rates compared to the control, but the growth rate tended to vary more with big growth spurts toward the last harvests (Fig 1d). One exception to this trend that the combinations of elicitors increased growth rate was the SA/GA/MJ combination. This combination showed consistently decreased growth compared to the control (Fig 1d). This combination got the lowest average growth rate in terms of slope (Table 3).

The plots for the control plant at week 8, GA 3 at week 8, MJ 2 at week 8, MJ 3 at week 10, GA/MJ treatment at week 8, and SA/GA/MJ at week 4 were outliers. These points were considered outliers because the total mass decreased, which would not have happened if the same plant had been monitored throughout the experiment. However, for this study, the plants harvested at each interval were not the same plants. These points were disregarded when drawing conclusions about the general trend; however, they were included for the calculation of slopes to balance the slope for the next point.

The exposure period study showed that the greatest growth rates occurred between harvest 4 and harvest 5 for the elicitor treated plants except for the MJ 1, MJ 3, and SA/MJ (MJ 3 has an outlier for the harvest 5 point). The SA 1 and SA 2 treatments showed the greatest growth between harvests 2 and 3.

Plotting each treatment group according to harvests showed that in general, the combination treatments had the greatest growth (except for SA/GA/MJ), followed in order by salicylic acid, gibberellic acid, methyl jasmonate, and control (Fig 2).

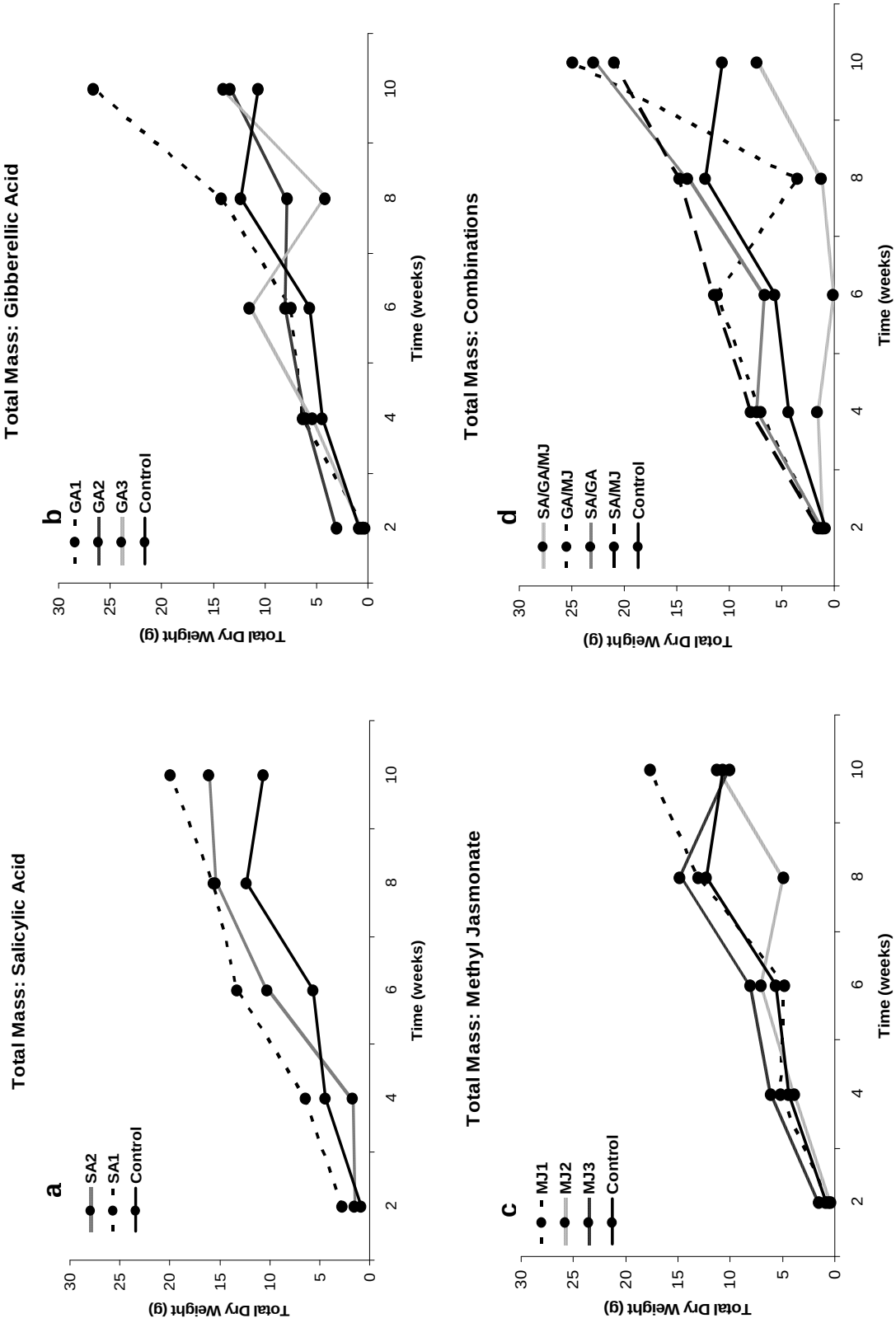

Figure 1. Total dry weight data for treatments with (a) salicylic acid, (b) gibberellic acid, and (c) methyl jasmonate and (d) combinations. Total dry weight combining shoots and roots were recorded after each harvest. Note the outlier points: control at week 8, GA 3 at week 8, MJ 2 at week 8, MJ 3 at week 10, GA/MJ treatment at week 8, and SA/GA/MJ at week 4. Legend: SA 1: 0.2 mM, SA 2: 1mM, GA1: 80µl, GA2: 400µl, GA 3: 2ml, MJ 1: 4µl, MJ 2 : 24µl, MJ 3 : 121µl

Table 3. Slopes for all harvests for each elicitor. Note bolded numbers represent the three highest average growth rates compared to the control as well as the lowest average growth rate.

Treatment	Harvest 1	Harvest 2	Harvest 3	Harvest 4	Average
Control	3.52	1.28	6.62	-1.62*	2.45
Gibberellic Acid 1	6.00	1.16	6.75	12.47	**6.59**
Gibberellic Acid 2	3.04	1.91	-0.17	5.55	2.58
Gibberellic Acid 3	4.86	6.05	-7.35*	9.89	3.36
Salicylic Acid 1	3.63	6.87	2.36	4.36	4.30
Salicylic Acid 2	0.18	8.59	5.13	0.67	3.64
Methyl Jasmonate 1	4.61	-0.44	8.29	4.58	4.26
Methyl Jasmonate 2	3.49	3.13	-2.15*	6.39	2.72
Methyl Jasmonate 3	4.63	1.99	6.71	-4.81*	2.13
SA/GA/MJ	0.53	-1.55*	1.17	6.13	**1.57**
GA/MJ	5.74	4.12	-7.65*	21.44	**5.91**
SA/GA	6.30	-0.75	7.40	8.92	**5.47**
SA/MJ	6.42	3.49	3.34	6.21	4.86

*Outlier points that were not included for observing trends.

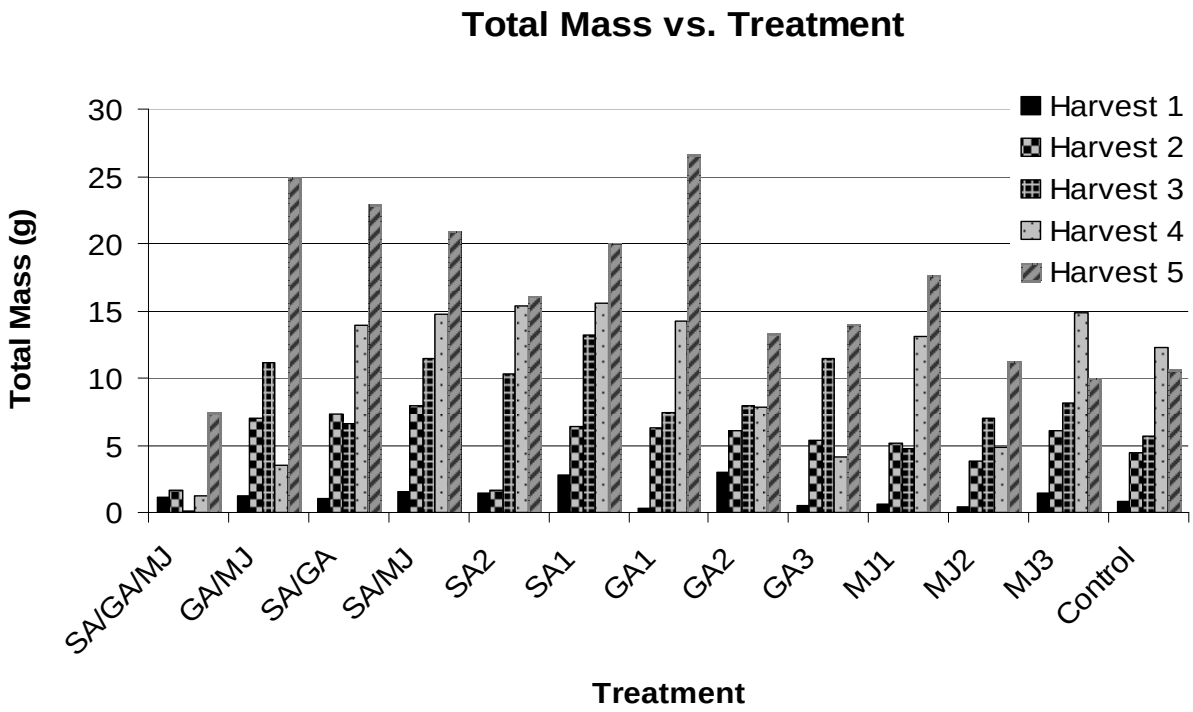

Figure 2. Total dry mass vs. treatment for all harvests. The combination treatments had the greatest growth (except for SA/GA/MJ, with the least growth) followed by SA, GA, MJ, and control in that order. Note each harvest took place at 2 week intervals with Harvest 1 at week 2.

DISCUSSION

The low yield of artemisinin in *A. annua* has been a serious constraint on the large-scale production of artemisinin-based drugs for treating malaria (Weathers 1994). Because of this constraint, finding ways to enhance the production of artemisinin using *A. annua* is an important advancement in fighting the global spread of malaria. The three elicitors, gibberellic acid, salicylic acid, and methyl jasmonate, have been shown to induce increases in *A. annua* growth and artemisinin levels in hairy root cultures (Smith et al. 1997, Baldi and Dixit 2007, Gao-Bin et al. 2009). This study investigated the effects of these chemicals on *A. annua* growth in hydroponic systems.

I hypothesized that all three chemical elicitors would increase *A. annua* growth compared to the control. The data (Fig 1) supports this hypothesis as a general trend. All three elicitors added individually to the hydroponic solution increased *A. annua* growth compared to the control, in agreement with my prediction. I rejected the hypothesis that the middle concentration, the concentration that was used in past tissue culture studies, would induce the greatest increases in growth. For salicylic acid and gibberellic acid, the lowest concentrations, which were one-fifth of the predicted concentrations, yielded the greatest increases in *A. annua* growth. For methyl jasmoante, the lowest and highest concentration treatments showed similar results.

The increase in plant growth with the addition of gibberellic acid in hydroponics can be explained by the hormone's ability to trigger growth factors and activate the defense pathway within the plant. Because of this, one of the immediate effects of gibberellic acid treatment on plants is an increase in shoot growth and height (Brian 2008). Gibberellic acid injected into drip irrigation lines in soil have been shown to significantly stimulate internode elongation of dwarf peas, and produce significantly taller plants than the control (Anderson et al. 1988). In addition, Yu et al. (2009) found that *Paris polyphylla* plants treated with gibberellic acid suffered less oxidative stress than the control. Avidan and Erez (1995) studied small rooted peach and nectarine plants in a hydroponic system with the addition of paclobutrazol (PBZ), which inhibits gibberellic acid biosynthesis. The inhibition of gibberellic acid's effects in a hydroponic system greatly reduced shoot to root ratio. This shows that gibberellic acid is an important component of promoting shoot growth, specifically in hydroponic systems. The addition of the hormone into the hydroponic solution to be absorbed directly at the roots is one explanation for the increase in

growth. Gibberellic acid's role in reducing stress for the plant may have also caused the increase in *A. annua* growth.

Brian (2008) describes comparing the effects of a range of gibberellic acid concentrations with *Pisum sativum* and found that low concentrations led to a greater increase in internode extension and leaf growth while the higher concentrations had less increases in growth, although the effects were more prolonged than the small dose. This supports the results of my study in which the GA 1 treatment showed the greatest growth in *A. annua*.

Salicylic acid as an elicitor in hydroponics significantly increased *A. annua* growth compared to the control. This suggests that salicylic acid acts on one of the plant growth pathways in *A. annua*. Salicylic acid's activation of the plant defense pathway through its signaling mechanism is one explanation (Zeng et al. 2009). Salicylic acid is a phenolic phytohormone involved in regulating plant growth, development, photosynthesis, transpiration, ion uptake and transport, and defenses against abiotic stresses such as UV radiation and ozone exposure (Gao-Bin et al. 2009). Also, Janda et al. (1998) found that hydroponic treatment of *Zea mays L.* plants with salicylic acid decreased the effects of chilling injury. In Metwally et al.'s study (2003), salicylic acid alleviated cadmium toxicity in barley seedlings. These results suggest that salicylic acid is involved in preventing plant cell damage in addition to the defense pathway. If addition of salicylic acid in hydroponics affected any one of these pathways, this could have caused the increase in *A. annua* growth.

An alternative salicylic acid pathway involves salicylic acid's inhibition of ethylene production. Raskin (1992) found that salicylic acid inhibits ethylene production in cell suspension cultures of various flowering plants. By blocking the production of ethylene, the salicylic acid inhibits plant senescence. In addition, salicylic acid reduces plant disease symptoms (Durner et al. 1997, Klessig et al. 2000, Raskin 1992). The combination of inhibiting senescence and reducing plant stress by preventing disease is a possible explanation for the increase in *A. annua* growth with salicylic acid treatment. I rejected the hypothesis that the concentration used in previous studies would yield the highest increase in growth because SA 1 yielded the highest increases in growth. This could be due to excess salicylic acid inhibiting ethylene production, eliminating the amount of naturally required levels of ethylene in the plant.

Methyl jasmonate as an elicitor in hydroponics significantly increased *A. annua* growth compared to control plants. This can be explained by the importance of jasmonates in regulating

plant responses to stress, UV light and ozone exposure, drought, insects, and other pathogens (Devoto and Turner 2005). Past studies found increases in plant growth with the addition of methyl jasmonate for *Lycopersicon esculentum* (Boughton et al. 2005) and *Arabidopsis* (Traw and Bergelson 2003). Also, exogenous methyl jasmonate treatment induced nicotine synthesis in hydroponically-grown *N. sylvestris* plants in Baldwin's study (1995). The resulting concentrations of nicotine protected tissues from insects and pathogens. In the case of *A. annua*, methyl jasmonate could have induced the production of artemisinin and other metabolites that protected tissues from pathogens, causing enhanced growth. In another study, treatment of *Lycopersicon esculentum L.* with low concentrations of methyl jasmonate significantly increased resistance to chilling injury and damage (Ding et al. 2002). This suggests that low doses of methyl jasmonate may prevent plant cell damage, improving growth.

The MJ 1 and MJ 3 treatments both had greater increases in growth compared to MJ 2, which I hypothesized would have the best growth. Based on this, conclusions can not be made on whether higher or lower concentrations of methyl jasmonate are more effective; however, past studies have shown that lower concentrations are more effective because high concentrations of methyl jasmonate act as an abiotic stress factor and can decrease nutrient uptake levels, causing a decrease in growth (Rossato et al. 2002, Wang et al. 2010).

The combination treatments (SA/GA, SA/MJ, GA/MJ) all showed increased growth rates compared to the control, with the exception of the combination of all three elicitors. The SA/MJ treatment consistently showed greater growth rates compared to the control, although a previous study found that salicylic acid inhibits plant response to methyl jasmonate (Traw and Bergelson 2003). This is most likely due to the function of both salicylic acid and methyl jasmonate as essential components of plant defense mechanisms against insect feeding, pathogen infection, and other environmental stress factors (Engelberth et al. 2003). The increases in growth observed with the SA/GA and GA/MJ treatments suggest, as presented by a previous study (Traw and Bergelson 2003), that the combination of gibberellic acid and an elicitor activating defense mechanisms (salicylic acid or methyl jasmonate) can act as effective elicitor combinations to increase *A. annua* growth.

Contrary to my hypothesis, the SA/GA/MJ combination treatment in hydroponics significantly decreased *A. annua* growth compared to the control. This result is most likely a result of plant chemical imbalances with the activation of multiple defense and growth pathways

induced by the three elicitors. Ro et al. (2006) found that adding multiple elicitors to a plant medium can decrease growth because it must respond to multiple stimuli with the addition of elicitors. In addition, salicylic acid negatively regulates the jasmonate-dependent pathway in plants in Traw and Bergelson's study (2003). The same study found that the combination of gibberellic acid and methyl jasmonate have a synergistic effect on plant growth in *Arabidopsis*; however, when salicylic acid was added, plant growth decreased. This suggests that when all of these chemicals were combined, the salicylic acid in combination with gibberellic acid and methyl jasmonate induced a decrease in the two elicitors' shoot elongation and plant defense activities.

The greatest growth rates generally occurred between harvest 4 and harvest 5 for the elicitor treated plants. This suggests that either *A. annua* has a growth spurt at around weeks 8 and 10, or that the three elicitors started to have an effect at around weeks 8 and 10. This could be a result of the decreased growth with multiple elicitors described by Ro et al. (2006) suppressing growth while the plants are small, and by week 8 and 10 the larger plant can now absorb and expel the unnecessary chemicals faster, leading to enhanced growth. The exceptions were the SA 1 and SA 2 treatments, which showed the greatest growth between harvests 2 and 3. This suggests that salicylic acid might be unique from the other treatments in that the elicitors are the most effective between weeks 4 and 6. This could be due to the inhibition of ethylene production that has a negative effect long-term as concluded by Raskin's study (1992).

Considering Cost

The addition of elicitors used in this study introduces a potentially cost-effective procedure for increasing *A. annua* growth and artemisinin production. The cost of the elicitors used in this study are relatively low (Table 4), and the chemicals used in the hydroponic solution cost only $1/week when bought in bulk. If additional studies show that these elicitors induce significant increases in *A. annua* growth, the addition of elicitors in hydroponics would be a cost-effective method. Comparing tissue culture expenses with a commercial hydroponic facility, growing *A. annua* in tissue culture would require significantly more labor and resources than a hydroponic facility that can be controlled easily with a computerized system (Jensen 1999, Govil and Gupta 1997). In addition, cultured plants produce low amounts of secondary metabolites compared to the intact plant and become inactive relatively quickly (Dicosmo and Misawa 1995).

Table 4. Costs per solution change for each treatment chemical. Note the hydroponic solution was changed every week during the study.

Chemical	Cost/gram (dollars/gram)	Totals/solution change (g/week)	Cost/week (dollars/week)
Salicylic Acid	0.05	6.35325	0.33
Gibberellic Acid	43.30	4.6	0.20
Methyl Jasmonate	7.50	230	1.73

Limitations

The limitations of this study include the lack of treatment replicates and the lack of similar studies for data comparison. Because this was a preliminary experiment, the goal was to test as many factors as possible including concentrations, combinations, and time. The experiments will definitely need to be repeated to strengthen inferences from the data. An ideal experiment would include at least 20 replicates of each treatment, that is, there would be at least 20 plant samples that are treated with the same chemical at the same concentration for the same amount of time (Wang et al. 2009). There are many external factors such as microbes and fungi in the hydroponic solution that were not considered; however, these mostly likely had minimal effects on the data, considering they would have affected each plant similarly.

Future considerations

The samples collected from my study were also prepared to be analyzed with HPLC (High Performance Liquid Chromatography) to measure the artemisinin concentration per gram of *A. annua* tissue. The samples were sent to my lab's collaborator in China (Dr. Chun-Zhao Liu at the State Key Laboratory of Biochemical Engineering) because the HPLC capabilities are not available at UC Berkeley. After the artemisinin data comes back from China, the differences between the artemisinin levels for each elicitor treatment will be analyzed. This will provide information on which elicitors in hydroponics are effective in increasing artemisinin levels in intact *A. annua* plants.

To increase cost-effectiveness, future studies may simply vary factors like temperature, amount of light, and pH to study *A. annua* growth rates because these would not require additional spending on chemical elicitors. In addition, as suggested by Brisibe et al. (2008), researchers can consider studying *A. annua* plants from different areas of the world, as described in Wallaart et al. (1999) and Simonnet et al.'s studies (2008), because they may have different

growth rates and shoot to root ratios. The results of my study showed that the GA 1, GA/MJ and GA/SA treatments had the highest growth rates. All of these treatments have gibberellic acid in common, which suggests that future studies may want to focus on studying the effects of gibberellic acid as an elicitor in hydroponics. For future studies focusing on elicitors, other elicitors can be experimented with, such as those used in Wang et al.'s study (2009), as well as different time intervals for harvests longer than 10 weeks to explore growth rates after 10 weeks. Also, the cost-effectiveness of the hydroponic method versus the tissue culture method will need to be further investigated in follow-up studies.

Conclusions

In conclusion, the addition of gibberellic acid, salicylic acid, and methyl jasmonate as individual and combination elicitors in hydroponics showed increased *A. annua* growth compared to the control. One exception was the combination treatment with all three elicitors, which showed a significant decrease in growth compared to the control. For salicylic acid and gibberellic acid, the optimal concentration for exposure was one-fifth the concentrations that were used in previous studies. For methyl jasmonate, no conclusions could be drawn about the optimal concentration. The interval between weeks 8 and 10 showed the greatest increase in growth. Because increases in *A. annua* growth can be correlated with higher artemisinin content, the methods used in this study will be one that may increase artemisinin yields per plant, creating a procedure through which artemisinin can be produced more efficiently with intact plants. Although this study was a preliminary experiment, the results show that adding elicitors in hydroponics is an important area of study.

The conclusions of this study will supplement the findings of a variety of experiments being done to increase the efficiency of artemisinin production. The results of these experiments will be used to design cost-effective methods to develop low-cost anti-malarial medications for distribution in the developing world. This will be particularly important with the projected increase in malaria cases in tropical countries. Manipulating *A. annua* plants to improve artemisinin production will lead to the increase in availability, quality, and affordability of anti-malarial medications around the world.

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REFERENCES

Abdin, M.Z., Israr, M., Rehman, R. U., and S.K. Jain. 2003. Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. Planta Medica 69: 289-299.

Adjuik, M., Babiker, A., Garner, P., Olliaro, P., Taylor, W., White, N. 2004. Artesunate combinations for treatment of malaria: meta-analysis. Lancet 363: 9–17.

Anderson, S.J., Franco-Vizcaino, E., and W.M. Jarrell. 1988. Dwarf pea response to gibberellic acid applied to soil through a drip irrigation system, and gibberellic acid biodegradation in soil. Plant and Soil 112: 289-292.

Anderson, S.J., Jarrell, W.M., and E. Franco-Vizcaino. 1988. Effects of concentration and treatment duration upon dwarf pea response to gibberellic acid root treatments in solution culture. Plant and Soil 112: 279-287.

Avidan, B. and A. Erez. 1995. Studies of the response of peach and nectarine plants to gibberellin biosynthesis inhibitors in a hydroponic system. Plant Growth Regulation 17: 73-80.

Baldi, A. and V.K. Dixit. 2007. Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Bioresource Technology 99: 4609-4614.

Baldwin, I.T. 1996. Methyl jasmonate-induced nicotine production in *Nicotiana attenuate*: inducing defenses in the field without wounding. Entomologia Experimentalis et Applicata 80: 213-220.

Balint, G. A. 2001. Artemisinin and its derivatives: an important new class of antimalarial agents. Pharmacology and Therapeutics 90: 261-265.

Boughton, A.J., Hoover, K., and G.W. Felton. 2005. Methyl jasmonate application induces increased densities of glandular trichomes on tomato, *Lycopersicon esculentum*. Journal of Chemical Ecology 31: 2211-2216.

Brian, P.W. 2008. Effects of gibberellins on plant growth and development. Biological Reviews 34: 37-77.

Brisibe, E.A., Uyoh, E.A., Brisibe, F., Magalhaes, P.M, and J.F.S. Ferreira. 2008. Building a golden triangle for the production and use of artemisinin derivatives against falciparum malaria in Africa. African Journal of Biotechnology 7 (25): 4884-4896.

Congpuong, K., Bualombai, P., Vanmairuroi, V., and K. Na-Bangchang. 2010. Compliance with a three-day course of artesunate-mefloquine combination and baseline anti-malarial treatment in an area of Thailand with highly multidrug resistant *falciparum* malaria. Malaria Journal 9:43-51.

Davies, M. J., Atkinson, C. J, Burns, C., Woolley, J. G., Hipps, N. A., Arroo R. R., Dungey, N., Robinson, T., Brown, P., Flockart, I., Hill, C., Smith, L., and S. Bentley. 2009. Enhancement of artemisinin concentration and yield in response to optimization of nitrogen and potassium supply to *Artemisia annua*. Annals of Botany 104: 315-323.

Devoto, A., and J.G. Turner. 2005. Jasmonate-regulated *Arabidopsis* stress signaling network. Physiologia Plantarium 123: 161-172.

Dicosmo, F., and M. Misawa. 1995. Plant cell and tissue culture: alternatives for metabolite production. Biotechnology Advance 13: 425-453.

Ding, C.K., Wang, C.Y., Gross, K.C., and D.L.Smith. 2002. Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. Planta 214: 895-901.

Duke, M.V., Paul, R.N., Elsohly, H.N., Sturtz, G., and S.O. Duke. 1994. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia anna L*. International Journal of Plant Sciences 155: 365-372.

Durner, J., Shah, J., and D.F. Klessig. 1997. Salicylic acid and disease resistance in plants. Trends in Plant Science 2: 266-274.

Engelberth, J., Schmelz, E.A., Alborn, H.T., Cardoza, Y.J., Huang, J., and J.H. Tumlinson. 2003. Simultaneous quantification of jasmonic acid and salicylic acid in plants by vapor-phase extraction and gas chromatography-chemical ionization-mass spectrometry. Analytical Biochemistry 312: 242-250.

Gao-Bin, P., Dong-Ming, M., Jian-Lin, C., Lan-Qing, M., Hong, W., Guo-Feng, L., He-Chun, Y., and B. Y. Liu. 2009. Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L. Plant Cell Reports 28:1127–1135.

Govil, S. and S.C. Gupta. 1997. Commercialization of plant tissue culture in India. Plant Cell, Tissue and Organ Culture 51: 65-73.

Houghton, J. 2005. Global Warming. Reports on Progressive Physics 68: 1343-1403.

Janda, T., Szalai, G., Tari, I., and E. Paldi. 1999. Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays L.*) plants. Planta 208: 175-180.

Jaziri, M., Diallo, B., Vanhalen, M., Homes, J., Yoshimatsu, K., and K. Shimomura. 1993. Immunodetection of artemisinin in *Artemisia annua* cultivated in hydroponic conditions. Phytochemistry 33: 821-826.

Jensen, M. .H. 1999. Hydroponics Worldwide. Acta Horticulturae 481:719-730.

Jones, J. B. 2005. Hydroponics: A Practical Guide for the Soilless Grower. 2nd Edition. Boca Raton, CRC Press.

Klessig, D.F., Durner, J., Noad, R., Navarre, D.A., Wendehenne, D., Kumar, D., Zhou, J.M., Shah, J., Zhang, S., Kachroo, P., Trifa, Y., Pontier, D., Lam, E., and H. Silva. 2000. Nitric oxide and salicylic acid signaling in plant defense. Proceedings of the National Academy of Sciences 97: 8849-8855.

Metwally, A., Finkemeier, I., Georgi, M., and K. J.Dietz. 2003. Salicylic acid alleviates the cadmium toxicity of barley seedlings. Plant Physiology 132: 272-281.

Mutabingwa, T.K. 2005. Artemisinin-based combination therapies (ACTs): Best hope for malaria treatment but inaccessible to the needy. Acta Tropica 95: 305-315.

Raskin, I. 1992. Role of Salicylic Acid in Plants. Annual Review of Plant Physiology and Plant Molecular Biology. 43: 439-463.

Resh, H. M. 2004. Hydroponic food production: a definitive Guidebook of soilless food-growing methods. 6th edition. Mahwah, NJ. New Concept Press, Inc.

Ro, D. K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C.Y., Withers, S.T., Shiba, Y., Sarpong, R., and J.D. Keasling. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440: 940-943.

Robert, A., Dechy-Cabaret, O., Cazelles, J., and B. Meunier. 2002. From mechanistic studies on artemisinin derivatives to new modular antimalarial drugs. Accounts of Chemical Research 35: 167-174.

Rossato, L., MacDuff, J.H., Laine, P., Deunff, E.L. and A. Ourry. 2002. Nitrogen storage and remobilization in *Brassica napus L.* during the growth cycle: effects of methyl jasmonate on nitrate uptake, senescence, growth, and VSP accumulation. Journal of Experimental Botany 53: 1131-1141.

Shukla, A., Faroogi, A., Shukla, Y.N., and S. Sharma. 1992. Effect of triacontanol and chlormequat on growth, plant hormones and artemisinin yield in *Artemisia annua* L. Plant Growth Regulation 11: 165-171.

Simonnet, X., Quennoz, M., and C. Carlen. 2008. New *Artemisia annua* hybrids with high artemisinin content. Asian Plants with Unique Horticulture Potential 769:371-375.

Smith, T. C., Weathers, P. J., and R. D. Cheetham. 1997. Effects of gibberellic acid on hairy root cultures of *Artemisia annua* growth and artemisinin production. In Vitro Cell Developmental Biology 33: 75-79.

Traw, M.B. and J. Bergelson. 2003. Interactive effects of jasmonic acid, salicylic acid, and gibberellin on induction of trichomes in *Arabidopsis*. Plant Physiology 133: 1367-1375.

Van Agtmael, M. A., Eggelte, T. A., and C. J. Van Boxtel. 1999. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. Trends in Pharmacological Sciences 20: 199-205.

Wagner, D. and Liebner, S. 2009. Global Warming and Carbon Dynamics in Permafrost Soils: Methane Production and Oxidation. Soil Biology 16: 219-236.

Wallaart, T.E., Pras, N., Beekman, A.C., and W.J. Quax. 1999. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chmotypes. Planta Medica 66: 57-62.

Wang, H., Ma, C., Du, Z., Wang, H., Ye, H., Li, G., Liu, B., and G. Xu. 2009. Secondary Metabolic Profiling and Artemisinin Biosynthesis of Two Genotypes of *Artemisia annua*. Planta Medica 75: 1625-1633.

Wang, H., Ma, C., Li, Z., Ma, L., Wang, H., Ye, H., Xu, G., and B. Liu. 2010. Effects of exogenous methyl jasmonate on artemisinin biosynthesis and secondary metabolites in *Artemisia anna L*. Industrial Crops and Products 31: 214-218.

Wang, J.W., Kong, F. X., and R. X. Tan. 2002. Improved artemisinin accumulation in hairy root cultures of *Artemisia annua* by (22S, 23S)-homobrassinolide. Biotechnology Letters 24: 1573-1577.

Wang, J.W. and R.X. Tan. 2002. Artemisinin production in *Artemisia annua* hairy root cultures with improved growth by altering the nitrogen source in the medium. Biotechnology Letters 24: 1153-1156.

Wang, J.W., Zheng, L.P., Zhang, B., and T. Zou. 2009. Stimulation of artemisinin synthesis by combined cerebroside and nitric oxide elicitation in *Artemisia annua* hairy roots. Applications of Microbiology and Biotechnology 85: 281-292.

Wang, Y., Zhang, H., Zhao, B., and X. Yuan. 2001. Improved growth of *Artemisia annua L* hairy roots and artemisinin production under red light conditions. Biotechnology Letters 23: 1971-1973.

Weathers, P.J., Cheetham, R.D., Follansbee, E., and K. Teoh. 1994. Artemisinin production by transformed roots of *Artemisia annua*. Biotechnology Letters 16: 1281-1286.

Yu, K., Wei, Jianrong, Ma, Q., Yu, D., and J. Li. 2009. Senescence of aerial parts is impeded by exogenous gibberellic acid in herbaceous perennial *Paris polyphylla*. Journal of Plant Physiology 166: 819-830.

Zeng, Q.P., Zeng, X.M., Yin, L.L., Yang, R.Y., Feng, L.L. and X.Q. Yang. 2009. Quantification of three key enzymes involved in artemisinin biogenesis in *Artemisia annua* by polyclonal antisera-based ELISA. Plant Molecular Biology Reports 27: 50-57.

Zeng, Q.P., Zeng, X.M., Yin, L.L., Yang, R.Y., Feng, L.L. and X.Q. Yang. 2009. Quantification of three key enzymes involved in artemisinin biogenesis in *Artemisia annua* by polyclonal antisera-based ELISA. Plant Molecular Biology Reports 27: 50-57.

Appendix A. Dry Weight for Each Treatment (in grams). Note that H denotes harvest, and each harvest occurred at two week intervals. All total masses were root weights and shoot weight combined in the results section.

Treatment	H1 Roots DW (g)	H1 Shoots DW (g)	H2 Roots DW (g)	H2 Shoots DW (g)	H3 Roots DW (g)	H3 Shoots DW (g)	H4 Roots DW (g)	H4 Shoots DW (g)	H5 Roots DW (g)	H5 Shoots DW (g)
SA/GA/MJ	0.3562	0.7562	0.405	1.239	0.012	0.081	0.511	0.75	2.217	5.175
SA/MJ	0.4219	1.0779	1.27	6.649	3.282	9.964	5.24	10.37	6.6	13.365
SA/GA	0.2482	0.7683	1.506	5.815	1.786	9.654	1.151	2.944	4.123	9.864
GA/MJ	0.3973	0.8853	1.702	5.316	2.39	8.746	1.021	2.461	9.25	15.667
MJ1	0.1639	0.4255	1.307	3.894	1.769	5.219	1.191	3.647	2.881	8.348
MJ2	0.0947	0.2699	0.917	2.937	1.479	4.186	3.871	8.416	2.415	8.255
MJ3	0.4453	1.0441	1.308	4.811	1.25	6.174	4.773	9.405	6.539	20.107
GA1	0.0613	0.2084	1.355	4.911	3.078	7.186	3.799	11.597	4.967	11.104
GA2	0.8943	2.134	1.39	4.674	1.292	5.281	4.054	9.915	6.7	16.192
GA3	0.1979	0.3299	0.697	4.689	2.521	5.449	1.778	6.018	3.013	10.33
SA1	0.8153	1.9312	1.441	4.931	3.066	8.345	4.468	10.278	4.781	16.178
SA2	0.3978	1.0958	0.333	1.34	1.113	3.649	3.8	9.255	5.282	12.352
Control	0.2197	0.6494	1.123	3.26	2.909	5.199	4.554	10.266	3.563	6.448