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Nutrient Deficiency in the Production of Artemisinin, Dihydroartemisinic Acid, and Artemisinic Acid in *Artemisia annua* L.

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Artemisia annua became a valuable agricultural crop after the World Health Organization recommended artemisinin as a component of ACT (artemisinin-combination based therapies) for malaria in 2001. A cloned, greenhouse-grown, *A. annua* (Artemis) subjected to an acidic soil and macronutrient deficit was evaluated for artemisinin production. Lack of lime (L) and macronutrients (N, P, and K) reduced leaf biomass accumulation. When L was provided (pH 5.1), the highest average leaf biomass was achieved with the "complete" (+N, +P, +K, and +L) treatment (70.3 g/plant), and the least biomass was achieved with the untreated (−N, −P, −K, and −L) treatment (6.18 g/plant). The nutrient least required for biomass accumulation per plant (g) was K (49.0 g), followed by P (36.5 g) and N (14.3 g). The artemisinin concentration (g/100 g) was significantly higher (75.5%) in −K plants when compared to plants under the complete treatment (1.62 vs 0.93%). Although the artemisinin total yield (g/plant) was 21% higher in −K plants, it was not significantly different from plants under the complete treatment (0.80 vs 0.66 g/plant). There were no marked treatment effects for concentration (g/100 g) or yield (g/plant) of both dihydroartemisinic acid and artemisinic acid, although higher levels were achieved in plants under the complete or −K treatments. There was a positive and significant correlation between artemisinin and both artemisinic acid and dihydroartemisin acid, in g/100 g and g/plant. This is the first report where potassium deficiency significantly increases the concentration (g/100 g) of artemisinin. Thus, under a mild potassium deficiency, *A. annua* farmers could achieve similar gains in artemisinin/ha, while saving on potassium fertilization, increasing the profitability of artemisinin production.

KEYWORDS: Macronutrients; nitrogen; phosphorus; potassium; pH; limestone; sweet wormwood

INTRODUCTION

Artemisinin-derived drugs have been shown to be effective against a variety of parasites that affect the health of humans and animals, such as *Plasmodium* spp. (*1*), *Coccidia* spp. (*2*), *Babesia* spp. (*3*), *Leishmania* spp. (*4*), *Neospora caninum* (*5*), and *Schistosoma* spp. (*6*, *7*), the latter living in the circulatory system of the host. By affecting either animals or humans, these parasites hinder economic development and often kill the parasitized species. Artemisinin-derived drugs have also been successfully used for the treatment of cancer (*8*, *9*) and, if approved by the Food and Drug Administration, will lead to an increased demand for artemisinin worldwide. *Artemisia annua* L. (Asteraceae), subsequently referred to as artemisia, is the only source of artemisinin but has also been tested as a valuable herbal tea in the control of malaria (*10*). When studied as a component of meat goat diet in vitro, artemisia did not affect the digestive microflora (*11*), suggesting its potential use as an alternative forage for small ruminants, and on the basis of its reported effects against *Schistosoma* spp., it could work as a natural means of controlling gastrointestinal parasites in small ruminants. Currently, however, the most important benefit for global health and economics is the agricultural expansion of artemisia cultivation areas to produce artemisinin to treat multidrug-resistant *Plasmodium falciparum*. Falciparum malaria afflicts over 300 million people and kills over 1 million children, under the age of five, yearly. Malaria-inflicted economic losses in Africa involve loss of labor, agricultural losses due to the debilitating disease, and poverty. These losses are estimated to be $12 billion a year in lost gross domestic product (*12*). Administration of artemisinin with other antimalarial drugs (artemisinin-based combination therapy, ACT) has been recommended by the World Health Organization (WHO) since 2001 wherever resistance to conventional antimalarial drugs has been observed (*13*). From 2001 to 2005, 53 countries in Africa, Asia, and South America have adopted ACT as the first- or second-line antimalarial where chloroquine is no longer effective (*13*). This recommendation caused the demand for artemisinin to

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increase, leading to supply shortages up to 2004 (*14*), although the situation has been recently reported as being under control (*13*).

To meet the demand generated by the WHO recommendation, production companies have expanded the artemisia cultivated area from 200 to approximately 1600 hectares in Kenya, Tanzania, and Uganda (*15*, *16*). This area is currently expanding with the cultivation of artemisia in Mozambique, Malawi, Zambia, Senegal, Ghana, Rwanda, South Africa, and Madagascar (*16*). In China, producers and associated farmers have announced their intent to increase annual production of artemisinin from 14 to 40 metric tons per year (*17*). It is estimated that the worldwide area needed to meet the current WHO-estimated demand for 266 million ACTs for 2006 is between 10000 and 11000 hectares, based on the estimate that 1 ha of artemisia produces artemisinin for approximately 25000 adult courses of ACTs (*18*).

The U.S. Agency for International Development is working in partnership with the WHO to increase artemisia cultivation, especially in Africa (*19*), and there is a pressing need to optimize artemisinin production per cultivated area, which will help meet the world demand of ACTs without intruding into areas originally cultivated with subsistence crops used to sustain a minimal nutritional level in African countries afflicted by malaria. However, there is little published information on individual nutrient requirements for growth and increased artemisinin production (*17*).

Increasing N application did not significantly affect artemisinin content or yield (*20*). In Madagascar, a field crop with three plants/m^2 that received 97 kg/ha of N increased dry leaf production from 2420 (control) to 4690 kg/ha, while the concentration of artemisinin dropped from 1.11 to 0.87%, but the increase in leaf production increased the artemisinin total yield from 27 to 41 kg/ha (*21*). Also, a mean 4.7 t/ha increase (19%) in total fresh plant biomass, cultivated in densities varying from 27.8 to 111.1 thousand plants/ha, was reported (*22*) with the addition of 67 kg/ha N, but the artemisinin concentration was not reported, and the total fresh plant biomass was reduced from 750 (at the lower density) to 275 g/plant (at the higher density). Boron deficiency inhibited flowering and decreased artemisinin concentration by approximately 50%, while a decrease of 25−30% in artemisinin concentration was reported for plants deficient in Fe, Mn, Zn, and Cu (*23*).

Strong genetic control of artemisinin has been demonstrated by broad-sense (*24*) and narrow-sense heritability (*25*), indicating that genes, rather than the environment, account for most of the artemisinin production of the plant. Approaches to increase artemisinin in artemisia under in vivo conditions have included water stress (*26*), the development of tetraploids (*27*), and foliar application of half-strength Hoagland solution containing gibberellic acid (GA_3) to sand-potted plants (*28*). The total artemisinin (g/plant) decreased in the first two studies. In the third study, the artemisinin concentration increased almost five-fold, from 0.14 (g/100 g) to 0.64% with 14 μM GA_3. However, artemisinic acid decreased from 0.59 to 0.21% at the same GA_3 concentration.

The production of secondary metabolites has been associated with increased levels of reactive oxygen species (ROS), which are byproducts of aerobic metabolism, or with biotic (insect, pathogen attack, and herbivores) and abiotic (drought, extreme temperatures, nutrient deprivation, and others) stresses (*29*−*31*). It has been hypothesized that ROS triggered by stress in artemisia are scavenged by dihydroartemisinic acid, which in turn is converted into artemisinin as a final, stable end product (*32*). In Africa, the main cultivated *A. annua* cultivars (Artemis and A-3) are provided by Swiss- (*25*) and German-based organizations, respectively, although some Nigerian growers are currently testing a cultivar (3M) from the University of Campinas, Brazil.

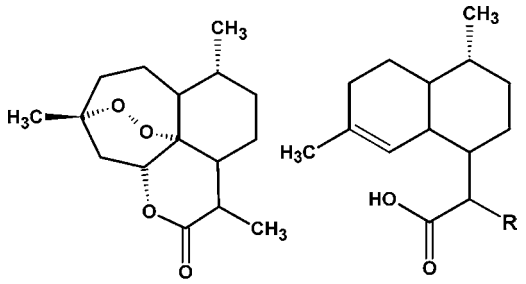

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Figure 1. Structures of artemisinin (**1**), artemisinic acid (**2**, R = CH_2), and dihydroartemisinic acid (**2**, R = CH_3).

The objectives of this study were to evaluate the effect of stresses associated with low pH and macronutrient deficiencies on the accumulation of leaf biomass, artemisinin (**Figure 1A**), and its precursors, dihydroartemisinic acid and artemisinic acid (**Figure 1B**), in *A. annua* plants grown on a dystrophic West Virginia soil under greenhouse conditions.

MATERIALS AND METHODS

Greenhouse Soil. The top 15 cm of a Gilpin (fine-loamy, mixed, mesic Typic Hapludult), Lily (fine-loamy, siliceous, mesic Typic Hapludult) series soil from a site (37° 40′ N, 81° 7′ W) in Cool Ridge, WV, was used for the greenhouse study. The soil was taken from an acidic, low-fertility, abandoned hayfield. After it was dried, the soil was passed through a 2 mm #10 metallic sieve (U.S.A. Standard Testing) before the addition of treatments. Plants were transferred to pots 3−4 days after treatment addition.

The aim of the soil regime was to establish low pH and low levels of available N, P, or K. To prepare the complete fertilizer and lime treatment, we added urea, $Ca(H_2PO_4)_2{\cdot}H_2O$, KCl, and $Ca(OH)_2$ sufficient to supply 100 mg/kg N, 115 mg/kg P, 150 mg/kg K, and 3.5 g/kg dolomitic limestone to 10 kg of air-dried soil to be used per pot. The limestone was characterized as containing 43% $MgCO_3$ and 54% $CaCO_3$ with 104% calcium carbonate equivalence and was passed through a #45 sieve (U.S.A. Standard Testing) to provide particles finer than 355 μm diameter for rapid reaction with the soil. The N-deficient treatment (−N) received all amendments except N. Treatments without P (−P), K (−K), and limestone (−L) were similarly prepared. The unamended treatment (untreated) did not receive N, P, K, or limestone. Plastic pots (13.5 L capacity) were used to contain the soil and allow collection of leachate samples. A hole was drilled at the bottom center of the pot for placement of a 13 mm fitting for leachate collection. Capillary matting (CAPMAT II, Hummert International, Earth City, MO) was prepared for the collection of leachates. A strip of matting 75 cm long and 6 mm wide was threaded through the hole in the bottom of the pot and through Tygon tubing to prevent drying and was covered with aluminum foil to deter algae growth. The end of the matting strip inside the pot was split into three 2 mm wide strips spread along the bottom of the pot, reaching to the pot wall. The bottom of the pot was then completely covered with a circular piece of capillary matting placed on top of the split pieces of matting. Each pot was filled with 10 kg of treated soil.

Plant Material. An artemisia plant of the cultivar Artemis (Mediplant, Conthey, Switzerland) was used as the source of clones for the experiment. This germplasm is representative of the *A. annua* grown

in Africa for production of artemisinin. On March first, 120 cuttings were dipped in Rhizopon AA#2 with 0.3% indole-3-butyric acid (Hortus USA Corp., New York, NY) and placed in a sand tray for rooting. Cuttings were misted for 12 s every 5 min in a growth chamber kept at 27 °C and 90% humidity, under a 15 h light (incandescent) regime. After 21 days, clones were transplanted into individual 7.6 cm pots filled with Pro-Mix BX growing medium (Premium Horticulture, Inc., Quakertown, PA). Twenty-four clones were used for the greenhouse experiment, and 90 clones were transferred to the field for comparisons on growth and artemisinin production.

Greenhouse Cultivation. On May 20, the 60 day old clones were separated into four blocks by size (20−25 cm in height) and transplanted individually into the prepared pots. To encourage root growth, pot-bound roots were severed by four vertical cuts on the outside of the growing medium.

Water was slowly added to each pot until field moisture capacity was reached. The pots were organized in a randomized complete block design with four blocks and four replicates per treatment. Pots were kept in a greenhouse at Beaver, WV (37° 46′ 30″ N and 81° 7′ 00″ W, 738 m of altitude) covered with two layers of K50 Clear 6 mm plastic (Klerks Plastic Product Manufacturing, Inc., Richburg, SC), with the temperature ranging from 19 to 35 °C. This cover allowed transmission of approximately 50% of UV rays, as measured by an Apogee SPEC-UV/PAR spectroradiometer (Apogee Instruments, Inc., Logan, UT).

Plants were photographed at regular intervals to document deficiencies as they occurred. Nitrogen deficiency symptoms were observed on June 27 [38 days after planting (DAP)], and an additional 1.0 g of urea (100 mg of N/kg of soil) was added to all treatments on July 11, except the untreated control and the −N treatment. On August 12, a supplement of 2.5 g of urea (250 mg N/kg soil) was added.

The soil moisture was measured twice a week using a Hydrosense digital soil moisture meter (Campbell Scientific Inc., Logan, UT) to calculate the volume of water needed to maintain field capacity. Distilled water was added three times per week. Subsamples of the leachate were collected for soil nitrogen analysis throughout the growing period, and the remaining leachate sample was returned to the pots during the watering process. Before urea applications, additional water was added to the buckets to leach out residual nitrogen, and the leachate was discarded.

Blocks 1−4 were harvested on August 24, 26, and 30 and September 1, 2005, respectively. The plants were cut 2.5 cm above the soil level, separated into stems, branches, and leaves, and oven dried at 40 °C for 24 h before they were weighed.

A composite soil sample consisting of five 0−15 cm deep subsamples was collected from each pot on December 6, 2005. These samples were air-dried and ground for nutrient analysis. Evaluation of pH was conducted on soil samples collected at the initiation of the experiment using a 1:1 soil:0.01 M $CaCl_2$ solution.

Field Cultivation. A field evaluation of artemisia growth and secondary metabolite production was conducted on a fertile Gilpin silt loam (fine-loamy, mixed, mesic Typic Hapludult) site at 37° 45′ N, 80° 59′ W (945 m of altitude). Approximately 90 clones of the same Swiss artemisia used in the greenhouse study were transferred to the field on June 1. Plants were spaced for a density of 10000 plants/ha (1 plant/ m^2) and received 45 kg of N, 20 kg of P, and 37 kg of K per hectare at planting (around the plant) and the same amounts 2 months after planting when deficiencies appeared. However, the light rains did not allow total incorporation of the second application. Plants were harvested in the first week of September, which coincided with the peak of artemisinin for this cultivar in Switzerland (*24*) and coincided with the termination of the greenhouse study.

Extraction of Artemisinin and Its Precursors. Whole artemisia plants were dried in a forced-air oven at 40−45 °C for 24−48 h after collection. Leaves were separated from the stems, passed through a metal U.S.A. Standard Test sieve #14 (1.4 mm opening), and stored at −20 °C until analysis (within 2 days).

Samples of approximately 500 mg of dried leaves were extracted once in 70 mL of petroleum ether by refluxing for 1.0 h. This shortened procedure, modified from previous work (*33*), resulted in the extraction of 95−98% of the artemisinin and its precursors artemisinic acid and dihydroartemisinic acid from the samples, as verified by a second extraction of three random samples. The supernatant was transferred to 150 mL glass beakers and evaporated to dryness in a hood. The following day, beakers were washed twice (10 mL each wash) with acetonitrile and filtered through a PTFE or nylon filter (0.45 μm) attached to a 5 mL syringe. These two washes were performed by rubbing acetonitrile on the sides and bottom of the beaker with a stainless steel spatula and by vortexing each wash around the tilted beaker. Recoveries for artemisinin and precursors were 95−97% with the first 10 mL wash, with the remaining being recovered in the second 10 mL wash. The samples were transferred to high-performance liquid chromatography (HPLC) flasks and analyzed in the same day that they were evaporated to dryness and reconstituted in acetonitrile. However, because the hood where samples were evaporated was not in total darkness and dihydroartemisinic acid photooxidizes to artemisinin under light, the dihydroartemisinin levels reported in this work were probably lower than the levels found in plants at the moment of sampling.

HPLC Analysis. Artemisinin, dihydroartemisinic acid, and artemisinic acid were quantified by an Agilent (Agilent Technologies, Inc., Pickerington, OH) HPLC (1100 Series) system composed of a G1379A degasser, a G1311A quaternary pump, a G1343A autosampler, a G1316A column oven set at 30 °C, containing a 150 mm × 4.6 mm i.d., 5 μm, Supelco Discovery C-18 column coupled to a G1315B diode array detector set at 194 nm, for artemisinic acid and dihydroartemisinic acid detection. A PL-ELSD 2100 (Polymer Laboratory, Amherst, MA) with the evaporator at 40 °C, nebulizer at 30 °C, and nitrogen flow of 1.2 L/min was used for artemisinin quantification. The mobile phase was isocratic and composed of 60% acetonitrile and 40% of a 0.1% acetic acid aqueous solution (pH 3.2) with a flow rate of 1.0 mL/min. Artemisinin, dihydroartemisinic acid, and artemisinic acid were calculated from a 10-point standard curve varying from 0.05 to 0.5 mg/mL for artemisinin and artemisinic acid and from 0.04 to 0.4 mg/mL for dihydroartemisinic acid, with a 10 μL injection per sample. Every batch of samples was coanalyzed with a 0.25 mg/mL artemisinin standard as a control sample to verify the accuracy of each detector. Plant samples that were too concentrated in artemisinin and exceeded the response capability of the ELSD (1100 mV) were reinjected at half volume (5 μL) before calculations.

Statistical Analysis. Analysis of variance and Pearson correlation evaluations were conducted using General Linear Model statistical SAS procedures (SAS Institute, Cary, NC). When the analysis of variance *F* test was significant at the 0.05 probability level, least significant differences were calculated to test differences among means. Differences and regressions discussed were significant at the 0.05 probability level unless otherwise stated.

Lead Nutrient Analysis. At the end of the experiment, leaf samples from both greenhouse and field plants were analyzed for macro- and micronutrients.

RESULTS AND DISCUSSION

The effects of low pH and macronutrient deficiencies on leaf biomass, concentration (g/100 g, w/w), and total yield (g/plant) of artemisinin, artemisinic acid, and dihydroartemisinic acid are discussed below. Because there are no pre-established levels of macronutrients deficiency for artemisia, plants were considered under nutrient stress after leaf symptoms were expressed (**Figure 2**).

Soil Fertility. A mean pH of 4.15 was measured in soils that received no limestone, while in the soil that received limestone, the mean soil pH was 5.1. Even with the addition of 450 mg/kg N, little or no excess ammonium or nitrate was detected in soil solution N for all treatments, except −L. The absence of soil solution N in the treatments where plant growth was vigorous indicated that soil N was readily uptaken by the plants, while the highest value (561) was recorded (**Table 1**) for the −L soil, where the low pH impaired N absorption by the plants. Phosphorus (P) addition of 125 mg/kg raised available soil levels to 28 mg/kg or higher. In the −P pots, soil levels were less than 8 mg/kg. All of the treatments receiving P showed available

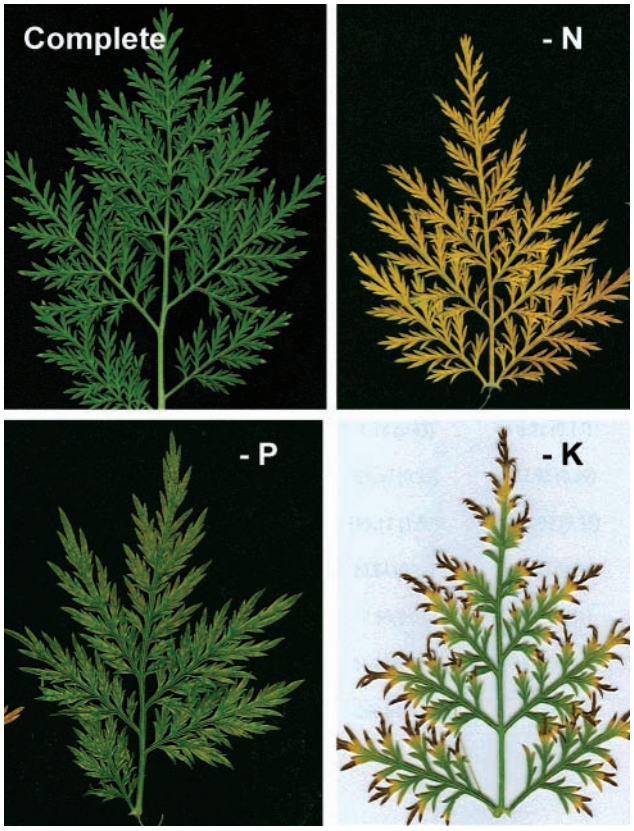

Figure 2. *A. annua* leaf appearances associated with the "complete" treatment (N, P, and K added) and with treatments where N, P, or K were excluded. Symptoms of nutrient deficiencies appeared approximately 40 days after the plants had been transplanted into pots containing a dystrophic Gilpin loamy (Typic Hapludult) soil under greenhouse conditions.

Table 1. Soil pH (0.01 M $CaCl_2$), Soil Solution N, Extractable P (Bray-I), and Exchangeable K, Ca, and Mg of the Soil Used in the Greenhouse Studies, Adjusted to Provide N, P, K, and Lime Deficiencies[a]

		mg/kg				
treatment	pH	soluble N	P	K	Ca	Mg
		greenhouse				
complete	5.08	2	28	82	439	192
–N	4.91	0	35	104	503	176
–P	5.06	63	6	93	488	189
–K	5.12	1	30	45	457	176
–L	4.20	561	51	116	184	55
untreated	4.10	0	8	49	102	26
LSD (0.05)	0.08	61	3	19	66	23
		field				
0–15 cm	6.0	NA	15	77	655	123
15–30 cm	5.9	NA	3	50	596	107

[a] Analysis of fertile soil where *A. annua* was field cultivated is provided for comparison.

soil levels of 28 mg/kg or above. A level of 30 mg/kg P is considered high (*34*), so all of the treatments receiving P had adequate or near-adequate levels. However, the adequate levels under field conditions may not be applicable in pot experiments where root expansion is limited by pot size. In the −P treatments, soil P levels were less than 8 mg/kg, well under the level of 15 mg/kg characterized (*34*) as low (**Table 1**).

Soils from −K pots had exchangeable K levels averaging 45 mg/kg, while soils from the complete averaged 82 mg/kg. The −L and the −N treatments maintained K levels of over 100 mg/kg (**Table 1**). The only treatments that maintained high K levels were the −L and the −N, where plant growth was so poor that the added K remained in the soil. The −K treatment provided approximately half the level of 100 mg of K/kg of soil, considered sufficient for growth (*35*). This indicates that soils assigned to −K were definitely deficient in K.

Table 2. Foliar Nutrient Concentrations of *A. annua* Plants Grown under Different Nutrient Regimes in a Greenhouse and under Fertile Field Conditions

	g/kg					mg/kg			
	N	P	K	Ca	Mg	Mn	B	Cu	Zn
				greenhouse					
complete	32.0	2.78	6.95	15.6	8.10	315	16	33	10
–N	18.9	5.10	29.30	18.7	7.70	349	48	53	21
–P	36.2	1.32	12.50	13.3	6.80	234	24	80	26
–K	40.0	4.60	3.45	12.6	11.2	195	17	47	11
–L	30.4	1.22	14.8	22.6	3.6	3110	31	18	70
untreated	22.9	1.82	9.45	20.3	6.3	1480	73	70	116
LSD (0.05)	2.4	0.39	1.81	2.48	1.36	159	7	6	13
				field					
average of six plants	46	3.6	23	8.9	4.4	180	86	24	25
SE	1.5	0.25	1.3	0.5	0.3	35	5.0	3.2	2.9

Soil magnesium (Mg) levels were 176−192 mg/kg where limestone was added but were 26 and 55 mg/kg on soils from the untreated and −L treatments, respectively. These Mg levels are below or at the level (50 mg/kg) at which some crops would respond to Mg additions (*34*), but in the untreated and −L soils, Mg deficiency would be overshadowed by the detrimental effects of low pH. Soil Mg levels were adequate in the soils receiving limestone, judging from Mg levels found in field soil with pH around 6.0 (**Table 1**).

A mean $CaCl_2$ soil pH of 4.15 was measured in −L treatments, while in the limed treatments mean soil pH was 5.1. The pH in the −L treatments was low enough to solubilize Al and Mn and create a hostile environment for root and shoot growth. The pH in the limed treatments was probably sufficient to overcome most limitations associated with soil acidity.

Leaf Nutrients. As compared to the complete treatment, foliar N decreased from 32.0 to 18.9 g/kg (−N), from 2.8 to 1.32 g/kg (−P), and from 6.95 to 3.45 g/kg (−K). Heavily fertilized field plants had foliar levels of N, P, and K of 46.2, 3.6, and 23.1 g/kg, respectively. Leaf deficiency symptoms expressed by −N, −P, and −K plants are shown (**Figure 2**). The levels of 19 g/kg N in −N treatment as compared to 32 g/kg in the complete and 46 mg/kg in the leaves from field plants indicated that the greenhouse −N plants were indeed deficient. Leaves from −P plants contained 1.32 g of P/kg, less than half of the 2.8 g/kg in the complete plants, while well-fertilized field plants had 3.6 g/kg. Foliar P in the complete treatment was probably near or within the adequate range, while the −P plants were deficient, considering the leaf symptoms (**Figure 2**).

Levels of foliar K were 3.5 g/kg in −K plants, 7.0 g/kg in complete, and 23 g/kg in fertile field plants (**Table 2**). The low foliar levels in the −K plants led to symptoms observed 38 days after transplanting (**Figure 2**). The well-fertilized field plants had about seven times more K than −K greenhouse plants and about three times more K than the complete plants, although

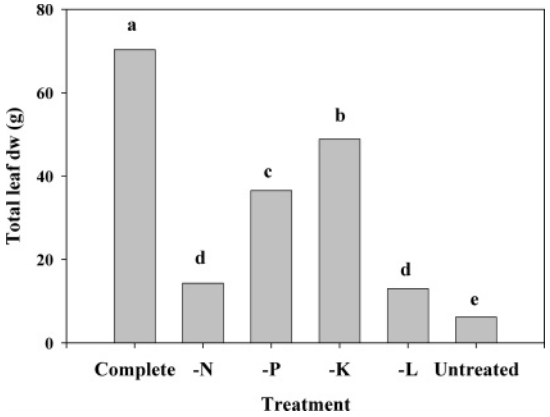

Figure 3. Total average leaf dry weights of *A. annua* grown in a greenhouse in pots containing a dystrophic Gilpin loamy (Typic Hapludult) soil under complete (with N, P, K, and lime) or missing one or all of the amendments.

Table 3. Comparison of Field and Greenhouse Soil Parameters, Leaf Fertility Levels and Biomass, and Concentrations of Artemisinin and Precursors of Cloned *A. annua* Grown under Both Environments[a]

parameters	field	greenhouse
soil type	A	B
pH	5.95	5.10
soil K (mg/kg)	77	45
leaf K (g/kg)	23.0	7.0
added K (g/plant)	3.74 (2×)	1.5
average leaf biomass (g/plant dry weight)	453.0[b]	70.0[c]
leaf/stem ratio	0.46[d]	0.51[c]
artemisinin (g/100 g)	0.69[e]	0.93[c]
artemisinic acid (g/100 g)	0.04[e]	0.036[c]
dihydroartemisinic acid	0.25[e]	0.16[c]

[a] Greenhouse parameters are given for the complete treatment only. A, Gilpin silt loam (fine-loamy, mixed, mesic Typic Hapludult); B, Gilpin (fine-loamy, mixed, mesic Typic Hapludult), Lily (fine-loamy, siliceous, mesic Typic Hapludult). [b] $n = 12$. [c] $n = 4$. [d] $n = 7$. [e] $n = 6$.

some of the foliar K in field plants may be attributed to luxury consumption. The complete plants may have been slightly low in K.

The mean foliar levels of Mn in −L treatments ranged from 1480 to 3110 mg/kg, about 10-fold higher than the foliar levels found in leaves of other treatments and in field plants (**Table 2**). Such high Mn levels in most crops are phytotoxic (*36*) and, in combination with levels of Al found at pH 4.15, can explain the stunted growth observed for −L plants, but Mn toxicity symptoms were not observed. Thus, the poor soil and fertilizer regime used here did establishe contrasts in pH and soil levels of N, P, and K.

The complete greenhouse treatment provided adequate or near-adequate foliar levels of nutrients for about 40 days after transplanting into large soil pots, after which foliar symptoms of deficiencies were noticed (**Figure 2**). On July 18, N deficiency was noticeable in all treatments, including complete. Thus, N had to be added to all treatments, except −N and untreated. Field plants, which received 45.0 g of 10−10−10 at planting and again after nutrient deficiency symptoms appeared (60 DAP) and were not collected in early September, remained in the field until December without showing macronutrient deficiency or toxicity. The field plants harvested in early September had foliar N, P, K, B, and Zn levels 44, 26, 32, 437, and 150%, respectively, higher than the greenhouse complete plants **(Table 2**). Leaf levels of Ca, Mg, Mn, and Cu were 75, 84, 75, and 37%, respectively, lower than the levels of the greenhouse complete plants (**Table 2**).

Leaf Biomass. Artemisia plants accumulated significantly more leaf dry weight in the complete treatment, with the least leaf biomass found in untreated plants. Considering leaf biomass production of the complete (70.3 g/plant) as 100%, average leaf biomass for each treatment was −K (69.5%), −P (52%), −N (20%), −L (18.5%), and untreated (8.8%) (**Figure 3**). The marked response of leaf biomass accumulation to the addition of lime supports previous reports that artemisia increases leaf biomass production with pH levels between 5.5 and 7.0, with little effect on artemisinin concentration (*37*). In the soil used in the greenhouse study, the most critical macronutrient for leaf biomass accumulation was N and the least critical was K. The omission of K resulted in maintaining 70% of the leaf production of the complete treatment, while N deprivation reduced leaf production to only 20.3% of the complete treatment. It has been established that N deficiency in sunflower severely reduced levels of cytokinins and growth (*38*). In the same study, these authors reported that K deficiency had the least effect on cytokinin concentration. That could explain why −K plants developed 70% of the leaf biomass of the complete plants. However, in the soil used for this greenhouse study, potassium was not totally absent. The soil K level in −K was about 50% of the K level in the complete, or 3.45 vs 6.95 g/kg, respectively (**Table 2**), and this may have allowed the plants under −K to accumulate considerable leaf biomass. Although K deficiency symptoms were visible in all plants under −K (**Figure 2**) 40 DAP, plants were not harvested for artemisinin analysis until about 103 DAP, at the termination of the study in late August. Field clones accumulated 23 g/kg of foliar K (**Table 2**) and never showed potassium deficiency, due to the availability of at about five times more K than it was available for greenhouse plants (**Table 3**).

Concentration of Artemisinin, Artemisinic Acid, and Dihydroartemisinic Acid. The artemisinin concentration (g/100 g w/w) was significantly higher in −K plants, with an average concentration 75.5% greater than that found in complete plants (1.63 vs 0.93%, respectively). There was no significant difference among the other treatments. Artemisinic acid and dihydroartemisinic acid concentrations (g/100 g) were not different ($p < 0.05$) across treatments, and concentrations varied from 0.025 to 0.038% for artemisinic acid and from 0.1 to 0.2% for dihydroartemisinic acid (**Figure 4**). Previous experiments have shown that providing complete fertilization to *A. annua* plants resulted in increased leaf biomass (*21*) and that N deficiency (*22*) or excess (*20*) decreased artemisinin in *A. annua*, but there are no studies relating P or K deficiencies with artemisinin concentrations. This is the first report that a nutrient deficiency can significantly increase both artemisinin concentration and artemisinin yield per plant. However, this is consistent with the hypothesis that secondary metabolites increase under stressful conditions.

Although nitrogen deficiency decreased leaf phenolic compounds and ROS in *Phaseolus vulgaris* L. (*39*), the results in this work showed only a slight decrease in artemisinin concentration for −N, −P, and −L plants (0.79, 0.75, and 0.71 g/100 g, respectively), as compared to the control (0.93%), and although the concentrationa of artemisinin in both untreated control and complete were similar (**Figure 4**), total artemisinin per plant (**Figure 5**) was low in all treatments, except in the complete and −K (0.66 and 0.80%, respectively), due to poor plant biomass production (**Figure 3**).

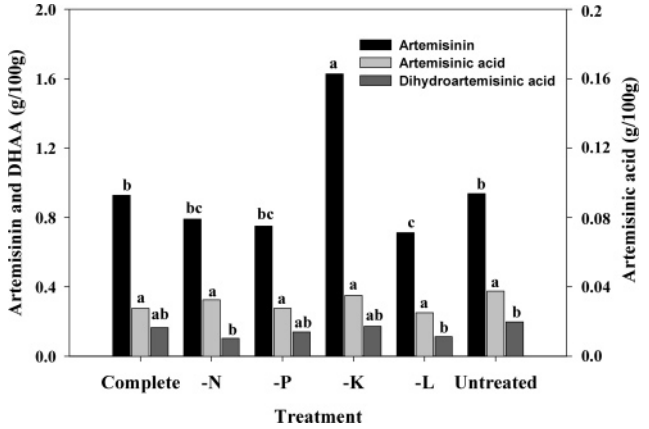

Figure 4. Artemisinin, dihydroartemisinic acid (DHAA), and artemisinic acid concentrations (g/100 g) in dried leaves of 4 month old *A. annua* plants, cultivated under greenhouse conditions in dystrophic soil deficient in lime or N, P, or K. Means for each parameter with the same letter are not significantly different at $P < 0.05$.

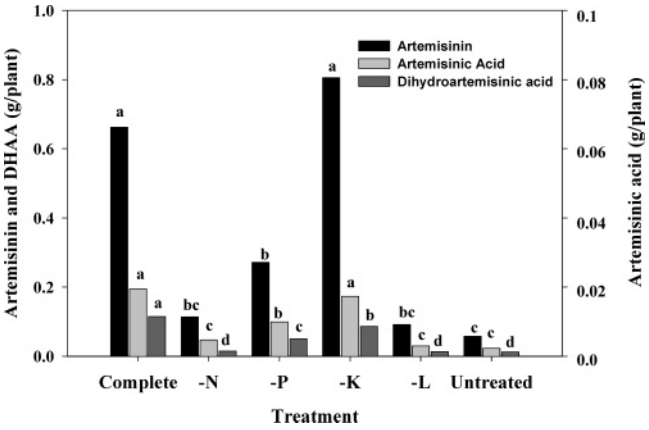

Figure 5. Total artemisinin, dihydroartemisinic acid (DHAA), and artemisinic acid (g/plant) for 4 month old *A. annua* plants cultivated under greenhouse conditions in dystrophic soil. Means for each parameter with the same letter are not significantly different at $P < 0.05$.

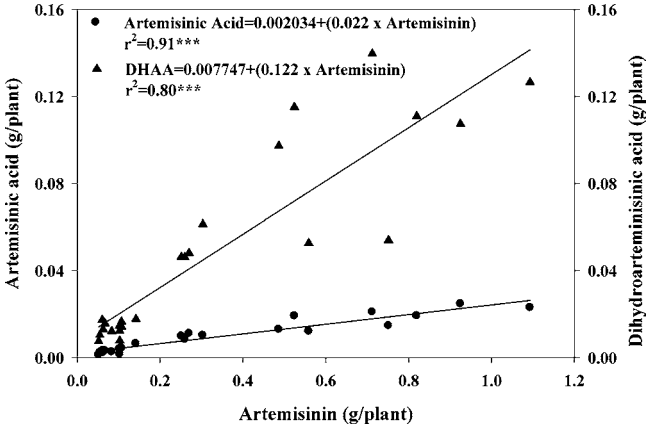

Figure 6. Relationship between artemisinic acid and dihydroartemisinic acid production to artemisinin (in g/plant) in 24 cloned *A. annua* plants grown under varying nutrient deficiencies. Significance levels were $p < 0.001$ and were expressed by "***" following the regression value (R^2).

Artemisinin (g/plant) in greenhouse plants correlated positively and significantly ($p < 0.001$) with the content of both artemisinic acid ($R^2 = 0.91$) and dihydroartemisinic acid ($R^2 = 0.81$), and both precursors reached higher contents in complete and −K plants (**Figure 6**). These results are opposite to the findings that GA_3 increased artemisinin about five-fold but decreased artemisinic acid in about three-fold (*28*). There is no evidence linking K deficiency, directly or indirectly, with increased artemisinin concentration, but some previous work might help us understand this observation.

First, there is evidence that the most important macronutrient for cytokinin accumulation in leaves of sunflower is N and that the least important is K (*38*). There is also the reported effect of the *ipt* (isopentenyl transferase) gene from T-DNA of *Agrobacterium* on chemical composition of transformed *A. annua* and that those plants had increased levels of cytokinin (2−3 fold), chlorophyll (20−60%), and artemisinin (30−70%) (*40*). These authors also reported a direct correlation between the concentrations of cytokinins, chlorophyll, and artemisinin. Other workers reported that, although under potassium stress, *Arabidopsis* leaves produced similar levels of chlorophyll as control plants (*41*). Artemisinin is produced in glandular trichomes, which have functional chloroplasts. Thus, cytokinins and chlorophyll could have an important role in artemisinin biosynthesis, although indirect, through the development of leaves and the photosynthetic secretory cells of glandular trichomes.

Second, potassium deprivation in *Arabidopsis* resulted in increased concentrations of ROS, and that ROS production is an early root response to potassium deficiency (*42*). Moreover, it is hypothesized that *A. annua* has adapted to convert dihydroartemisinic acid into artemisinin as a protection mechanism against the build-up of ROS (*32*). Given that dihydroartemisinic acid spontaneously converts to artemisinin by reacting with ambient oxygen and assuming that high concentrations of ROS in *A. annua* are scavenged by dihydroartemisinic acid and stabilized as artemisinin, it is plausible to assume that K deficiency sensed by the roots triggered the production of ROS in this study and that ROS led to increased artemisinin accumulation.

Third, K deficiency has been linked with increased amounts of putrescine in *Pinus sylvestris* (*43*) and of arginine in *Arabdopsis thaliana*, leading to an up to 20-fold increase in putrescine levels (*41*). A lack of potassium in *Coffea arabica* has also led to increased concentration of caffeine by 12% when compared to the control (24.5 vs 21.9 g/kg) (*44*). However, no increases in terpenes, other than those found in pine resin, have been associated with potassium deficiency (*45*). These authors reported that over 90% of the monoterpene cyclases lost activity in media lacking monovalent cations, potassium being the most important. Although these terpene cyclases are different from the ones found in glandular trichomes (*45*), potassium is an important cofactor for many enzymes. In the mevalonic acid pathway, FPP can be funneled into sesquiterpenes such as artemisinin and its precursors or can go toward the production of monoterpenes and sterols. Assuming that potassium is more important for enzymes involved in the biosynthesis of monoterpenes and sterols, the pathway could be shunted toward the production of artemisinin.

Thus, the facts that moderate potassium deficiency does not critically affect cytokinin (*38*) or chlorophyll (*41*) accumulation in leaves, allowing for reasonable plant growth and leaf trichome development; the increase in cytokinins and artemisinin are directly correlated (*40*); K deficiency can trigger the formation of ROS, speculated to increase artemisinin production (*32*); or the assumption that K deficiency could shunt FPP toward artemisinin production could, together, explain the significant

increase in artemisinin observed here. Although we cannot pinpoint the link between K deficiency and increased artemisinin production at this moment, this study indicates that K deficiency can be an important approach to investigate the physiology and biochemistry of artemisinin and other secondary metabolites accumulation such as cytokinins and chlorophyll or compounds generated by stress, such as ROS. Also, these results could be applicable to increase field artemisinin production on a short run rather than relying on the long-run approach involving genetically engineered plants or microorganisms.

Total Yield of Artemisinin, Dihydroartemisinic Acid, and Artemisinic Acid. The total yields of these compounds (g/plant) were calculated by multiplying their average concentrations (g/100 g) by the total leaf biomass (g) of the plants in each treatment. Although the −K treatment reduced the artemisia leaf biomass accumulation to 69.5% of the complete treatment, it increased the total artemisinin content of the plants 21% as compared to the complete (0.80 vs 0.66 g/plant), although this increase was not significant at $p < 0.05$. Artemisinic acid contents (g/plant) were significantly higher in the complete and −K treatments (0.019 and 0.017%, respectively), followed by −P (0.009%), and varied from 0.002 to 0.004% for all other treatments. The dihydroartemisinic acid content was significantly ($p < 0.05$) higher for the complete (0.11 g/plant), followed by −K (0.09 g/plant). The dihydroartemisinic acid content varied from 0.01 to 0.05 g/plant for the other treatments (**Figure 5**). The field leaf production of the cloned Mediplant used was at least twice as high as the 100−200 g of dried leaves per plant reported for the same Artemis cultivar in Central Africa and higher than the 250−300 g/plant reported for Europe (*46*). These greenhouse results suggest that the plant can grow successfully in poor soils provided that adequate nitrogen, phosphorus, some potassium, and sufficient limestone are present with a pH of 5.1 or higher. Greenhouse plants achieved 70% of their growth potential under those conditions. More importantly, although diminished in their growth, the total artemisinin production (g/plant) achieved with the −K treatment was 21% higher, although not significant (**Figure 5**), than that of plants receiving complete fertilization (0.8 vs 0.65 g/plant).

Greenhouse vs Field Clones. Under West Virginia field conditions, artemisia cloned plants, grown in soil similar to the one used in the greenhouse study, with a pH of 5.95 and receiving 90 kg of N, 39 kg of P, and 74 kg of K/ha in two applications, produced an average of 450 g of dry leaves/plant (4.5 tons/ha). The average field artemisinin concentration was 0.69%, based on three cloned plants harvested on September 2 and three cloned plants harvested on September 9, 2005, while the average artemisinin concentration for greenhouse clones submitted to the complete treatment was 0.93% (**Table 3**). The cloned field-grown plants, cultivated on a similar soil as greenhouse plants, with a pH of 5.8, and with more available N,P, and K than the greenhouse-grown clones, had no nutrient deficiency or toxicity symptoms but produced slightly less artemisinin (0.69 vs 0.93%) than greenhouse complete plants, although the concentrations of artemisinic acid and dihydroartemisinic acid were similar (**Table 3**). This decreased artemisinin concentration could be a result of excess N fertilization at field levels, as reported from a study in Madagascar (*21*). Cloned field plants produced an average of 450 g of leaf dry weight per plant. Considering 10000 plants per hectare, at 0.7% artemisinin, one could expect 31 kg of artemisinin/ha. Considering values given in **Table 3** for field-grown plants, the estimated production of dihydroartemisinic acid would be 11.25 kg/ha and that of artemisinic acid would be 1.8 kg/ha. These two precursors can be converted to artemisinin, further increasing artemisinin final yield.

Assuming that K deficiency would increase average artemisinin per plant by 21% in field plants, as it did in this greenhouse study, and assuming a field yield of 4 tons of leaves/ha, containing 0.7 g/100 g artemisinin, we can estimate that a soil low in K will lead to an increase of about 5.9 kg artemisinin/ha. Considering the estimated price of U.S. $20.00/kg (intrinsic value of artemisinin in dry leaves) (*47*), a 21% increase in artemisinin/plant would amount to about U.S. $120.00 extra per hectare to the farmer, although the potential profit from the conversion of artemisinic and dihydroartemisinic acids into artemisinin is not considered in this price. In addition, further savings can be achieved by decreasing potassium input.

In Africa, fertilizers are 2−6 times more expensive than in Europe, North America, and Asia (*48*), and small-scale farming has depleted 22 kg of N, 2.5 kg of P, and 15 kg of K per hectare over the past 30 years, an annual loss equivalent to U.S. $4 billion in fertilizer (*48*). China imported 90% of the potassium required in 2001/2002 amounting to 4.0 million metric tons, second to the 5.3 million tons imported by the United States in that period (*49*). Thus, any increase in the agricultural production of artemisinin from *A. annua* cultivated under low potassium fertilization should be profitable not only in Asian and African countries but also in other countries interested in artemisia cultivation for artemisinin production.

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