Artemisia annua Allelopathy as a Potential Striga Control Mechanism

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Abstract

*Artemisia annua* L. produces the anti-malarial drug, artemisinin, which is a major component of the current malaria treatment. Artemisinin has also been shown to inhibit growth and germination of other plants. The parasitic plant *Striga* devastates crops in Africa, threatening food security and increasing poverty. *A. annua* field debris could be used to inhibit *Striga* but could harm the soil’s viability for food crops. If *A. annua* does not harm soil viability, farmers could alternate a medicinal crop of *A. annua* with their subsistence food crops. The effects of *A. annua* on bush beans (*Phaseolus vulgaris*), corn (*Zea mays*), *Rhizobium* spp. and soil microbes were measured. Although pure artemisinin inhibited *Striga* seed germination, it did not affect the germination rate of either crop plant. Dried *A. annua* leaves caused a delay in germination of bush beans but not corn and the effect was temporary. While *A. annua* altered the microbe composition of the soil, it did not affect crop growth or the nodulation of beans by *Rhizobium*.

Acknowledgements

The author greatly appreciates the guidance of his advisor, Professor Pamela Weathers, and the help of her lab team, particularly Khanhvan Nguyen and Melissa Towler. Prof. Michael Timko of the University of Virginia and his student Kan Huang also made valuable contributions to this project. WPI’s Office of Undergraduate Studies arranged the Summer Undergraduate Research Fellowship that partially funded the author’s involvement in this work.
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1. Introduction

Sub-Saharan Africa is a region where countries are plagued by famine, war, disease and extreme poverty; it is difficult to develop the area because there are numerous obstacles to agriculture and diseases reduce or disable much of the workforce (Sachs and Malaney 2002). Malaria, caused by the parasite Plasmodium (*P. falciparum* or *P. vivax*), is a debilitating disease present in most tropical areas; in 2008 there were over 60 million reported cases of malaria in the African Continent and over 170 million cases worldwide (World Health Organization 2010). The current treatment uses a derivative of artemisinin (AN) with another antimalarial drug as an artemisinin combination therapy (ACT). While effective, ACT is still relatively too expensive to make treatment available to enough people; indeed, millions of people die of malaria each year (Gollin and Zimmerman 2007).

![Fig 1 Artemisia annua (left) and the medicinal sesquiterpene lactone, artemisinin (right).](image)

AN is a sesquiterpene lactone (Figure 1) that is produced by the plant *Artemisia annua*. African farmers themselves may be able to grow their own *A. annua* for sale as a medicinal cash crop to supplement their income (Weathers et al. 2010). Health organizations can then use this new local supply of AN to bolster their combined therapy regimens. One concern with an increase in *A. annua* production on farmland is that AN and artemisinic compounds have been known to be toxic to some plants (Duke et al. 1987; Lydon et al. 1997), animals, microbes and algae (Jessing et al. 2009). This toxicity could potentially harm crops or alternatively be harnessed as a useful tool against weeds and insect pests.

Compounding the hardship caused by malaria is widespread loss of crops to various species of the parasitic angiosperm, *Striga*, which claims a significant portion of the Sub-Saharan African subsistence and cash crops, and each year threatens the food security of 100 million people (Berner et al. 1995). Recently, control of *Striga* among certain crops through the use of intercropping with antagonists to *Striga* has been implemented in many farming communities (Amudavi et al. 2009). It is an enticing idea to think that the toxicity of *Artemisia annua* could be employed against *Striga* without harming crops.
To determine if this is possible, a series of experiments were conducted to test the effects of *A. annua* cultivation on staple crops, corn (*Zea mays*) and green beans (*Phaseolus vulgaris*), and on the microbiological composition of soil, including the nitrogen-fixing bacteria *Rhizobium spp.* that form nodules on bean roots. Further experiments to test the effects of *A. annua* cultivation and AN itself on the parasitic weed *Striga* are being conducted by an associate with access to the necessary Bio-Safety Level 4 facilities. The results of these trials will show whether it is possible to control a pest with a medicinal plant and whether it is safe to grow large amounts of the medicinal plant in a region that has great need of it.

2. Background

2.1 *Striga*  
Species within the genus *Striga*, a parasitic angiosperm (Fig 2), negatively affect crop productivity in Sub-Saharan Africa (Tsanuo et al. 2003). *Striga* species affect nearly all staple crops in the area: corn, sorghum, soybean, millet and rice, and the feed crop, cowpea (Table 1). Each *Striga* plant produces thousands of seeds which can remain dormant in the soil for up to twenty years and can travel far with the wind and human activity (Khan et al. 2002). It is estimated that *Striga* affects the livelihoods of over 100 million people in Sub-Saharan Africa, causing widespread crop loss every harvest (Khan et al. 2009).

![Fig 2 A sorghum field infested with *S. hermonthica* (left); image taken from Western Seed Company (2005). Some strigolactones (right) that are known to induce germination are strigol, orobanchol and sorgolactone (Matusova et al. 2005). GR 24 is a synthetic lactone that mimics strigolactones. Image adapted from Matusova et al. (2005).](image)

*Striga* seeds lie dormant in the soil until host plants grow roots nearby; the growing roots secrete secondary metabolites called strigolactones (Fig 2) which induce the parasite’s germination (Lopez-Raez et al. 2009; Matusova et al. 2005). Strigolactones are thought to be used by plants to signal arbuscular mycorrhizae to grow and establish symbioses and to limit root branching within the plant; *Striga* exploits this signal for its own seeds to grow and parasitize the host (Lopez-Raez et al. 2009). Once the seed
germinates and its radicles develop, the haustoria develop; these attach to the host plant roots and penetrate it. A vascular connection to the host is formed through which the young parasite drains water and nutrients from the host (Fig 3). The young plant then uses these resources to grow and finally emerge from the ground, flowers and produces new seeds (Botanga and Timko 2005).

When *Striga asiatica* was accidentally introduced to the United States, it was brought under control through quarantines, restriction of movement of farm equipment, and use of herbicides and ethylene (Eplee 1981). Ethylene induces the seeds to germinate without the presence of a host, leading to death (Musselman 1980; Eplee 1981). Although reasonable in wealthier developed countries, such practices are too expensive, harmful, or ineffective in impoverished Sub-Saharan Africa. Other control techniques involve the use of plants to inhibit *Striga* by means of early germination and growth inhibition, for example, Sudan grass is known to work against *S. hermonthica* through allelopathy (Musselman 1980).

**Table 1** Selected *Striga* species that are active in Africa and their host food and commercial crops.

<table>
<thead>
<tr>
<th>Striga Species</th>
<th>Affected Crops</th>
<th>Selected Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. hermonthica</em></td>
<td>Millet, sorghum, rice, corn</td>
<td>Musselman 1980; Botanga and Timko 2005</td>
</tr>
<tr>
<td><em>S. gesnerioides</em></td>
<td>Cowpea, tobacco, sweet potato</td>
<td>Musselman 1980</td>
</tr>
<tr>
<td><em>S. asiatica</em></td>
<td>Millet, sorghum, rice, corn, sugarcane</td>
<td>Musselman 1980; Botanga and Timko 2005</td>
</tr>
<tr>
<td><em>S. euphrasioides</em></td>
<td>Sorghum, sugarcane</td>
<td>Musselman 1980</td>
</tr>
</tbody>
</table>
Fig 3 The life cycle of Striga, which spends most of its time underground and only germinates when it senses its host’s chemical cues, strigolactones. Image taken from Timko (2010).

2.2 Push-Pull Technology
During the development of a system to fight stem-boring moths, researchers discovered a new control for Striga. Push-Pull technology (Fig 4) is a means to protect corn from stem-borers by growing corn with two additional plant species. The first is a trap plant (pull) such as Pennisetum purpureum (Napier or Uganda grass) or Busseola fusca; the insects lay their eggs in the trap plant instead of the crop, this plant is grown away from the corn. The corn is intercropped with a repellent (push) plant, such as Melinis minutiflora (molasses grass) or Desmodium uncinatum (silverleaf); these plants discourage the pests from approaching the corn.

A side benefit to the protection of corn from the stem borers was the observation that D. uncinatum significantly reduced S. hermonthica infestation, which added to the value of the D. uncinatum cattle feed crop (Khan et al. 2002). As proposed by Khan et al. (2002), D. uncinatum may exert its control via three means. First, by providing shade from the sun, D. uncinatum has been shown to reduce infestation. Second, the plant fixes atmospheric nitrogen, which also reduces infestation. Last, the plant induces suicidal germination and inhibits radicle growth in Striga through allelopathy.
2.3 Allelopathy

Allelopathy is the inhibitory effect one plant exerts upon another through chemicals it produces and releases into the environment (Inderjit et al. 2005). *Desmodium* is capable of inhibiting *Striga* through the production of allelochemicals, numerous secondary metabolites that are believed to be isoflavones that are produced in its root hairs (Tsanuo et al. 2003). Other plants are capable of producing similar secondary compounds as well, which may themselves prove effective against *Striga* (Hooper et al. 2009). Indeed, lactones of certain plants may have an effect similar to strigolactones that induce germination in *Striga*.

Of interest to this project are the sesquiterpene lactone, artemisinin (AN), and other, as of yet unidentified, potential allelochemicals present in the plant *Artemisia annua* L. AN is highly phytotoxic, even to *A. annua* (Duke et al. 1987) and has shown an allelopathic effect on redroot pigweed and soybean (Lydon et al. 1997). Indeed, *A. annua* dry leaves have been shown to inhibit weed infestation in the field (Bohren et al. 2004). AN may act by interfering with mitosis even at very low concentrations in lettuce and ryegrass (Dayan et al. 1999).
2.4 *Artemisia annua* and Artemisinin

Artemisinin is produced in the glandular trichomes of *A. annua* (Fig 1) and is still one of the best known treatments against malaria (Mutabingwa 2005). Unfortunately *A. annua* produces so little AN, at best about 2% dry weight, that very large amounts of the plant are required to satisfy the world’s need for the antimalarial drug as ACT (Mutabingwa 2005; Jessing et al. 2009).

In any case, more ACT is needed in Sub-Saharan Africa, which suffered some 60 million reported cases of Malaria in 2008 (World Health Organization 2010). Farmers in Africa could grow it in rotation with food crops to sell as a medicinal cash crop. *A. annua* would then supplement their meager incomes. Given *A. annua*’s allelopathic activities, it is possible that it could help control *Striga*, thereby making it doubly useful to the farmer. *A. annua* is not normally grown alongside other crops, however, and thus its potential negative interactions require further investigation.

2.5 Toxicity of *A. annua* to Other Organisms

Artemisinin is a biologically active compound that can harm a wide variety of organisms (Table 2). Its toxicity makes it useful against malaria and could help control weeds as persistent as *Striga*. However that same toxicity could lead to adverse environmental effects and loss of soil viability. For example, Jessing et al. (2009) found that AN can be leached into the soil after rain and that although most of it degrades quickly, the rest can persist at detectable levels for lengths of time comparable to those of commercial herbicides. Furthermore, AN was also shown to repel earthworms and was toxic to duckweed and green algae (Jessing et al. 2009). Lydon (1997) showed that dried *A. annua* leaves inhibited redroot pigweed germination and slightly affected the germination and growth of corn, lambsquarter and soybean. Although Morvillo et al. (2011) found a positive effect on *Bradyrhizobium* nodules in soybean roots under field conditions, to my knowledge, the impact of growing *A. annua* on the microbial community of the soil has not been widely studied.
Table 2 Some of the organisms that have been shown in the literature to be either inhibited or repelled by artemisinin.

<table>
<thead>
<tr>
<th>Organism Affected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Artemisia annua</em></td>
<td>Duke et al. 1987</td>
</tr>
<tr>
<td><em>Lactuca sativa</em> (lettuce)</td>
<td>Duke at al. 1987;</td>
</tr>
<tr>
<td></td>
<td>Lydon et al. 1997;</td>
</tr>
<tr>
<td></td>
<td>Dayan et al. 1999;</td>
</tr>
<tr>
<td></td>
<td>Jessing et al. 2009</td>
</tr>
<tr>
<td><em>Glycine max</em> (soybean)</td>
<td>Lydon et al. 1997</td>
</tr>
<tr>
<td><em>Zea Mays</em> (corn)</td>
<td>Duke et al. 1987</td>
</tr>
<tr>
<td><em>Amaranthus retroflexus</em> (redroot pigweed)</td>
<td>Duke et al. 1987;</td>
</tr>
<tr>
<td></td>
<td>Lydon et al. 1997</td>
</tr>
<tr>
<td><em>Chenopodium berlandieri</em> (lambsquarter)</td>
<td>Lydon et al. 1997</td>
</tr>
<tr>
<td><em>Lolium multiflorum</em> (ryegrass)</td>
<td>Dayan et al. 1999</td>
</tr>
<tr>
<td><em>Eisenia fetida</em> (earthworm)</td>
<td>Jessing et al. 2009</td>
</tr>
</tbody>
</table>

3. Methodology

3.1 *Artemisia annua*

The *Artemisia annua* cultivar that was used was PEG01 Chinese, which was received in 2007 as a gift from Prof. Chun Zhao Liu of the Chinese Academy of Sciences in Beijing. The plants were grown from F2-F4 seed derived from the PEG01 plants and grown in a growth chamber at 25°C under continuous GE Cool White fluorescent lighting with intensity at soil level of 32µmol m⁻² sec⁻¹ (under edge of light bulb) to 42µmol m⁻² sec⁻¹ (under middle of light bulb) to inhibit flowering. Once the plants reached a height of approximately 0.5m they were harvested and dried under the lights in the growth chamber for four to seven days. Once dry, the leaves were removed, sifted through a 1mm sieve and were manually pulverized. The dried leaves were stored in re-sealable plastic bags in the dark at room temperature (~22°C).

3.2 Corn and Bean Germination and Growth

All corn and bean plants were grown in a 25°C growth chamber under continuous GE Full Spectrum fluorescent lights with intensity at soil level of 32µmol m⁻² sec⁻¹ (under edge of light bulb) to 42µmol m⁻² sec⁻¹ (under middle of light bulb). Watering occurred twice each week, at which time the pots were rotated around the pan that contained them to normalize the light intensity. All plants were grown in MetroMix 360 (Sungro Horticulture Canada Ltd.) potting mix, hereafter referred to simply as “soil”.

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The effect of *A. annua* growth was tested on corn (*Zea mays* F1 Hybrid, Pinetree Garden Seeds) and bush beans (*Phaseolus vulgaris*, “Blue Lake 274” cultivar, Pinetree Garden Seeds) in two ways. First, corn and beans were each grown in soil that had been used to grow *A. annua*, to test the effects of the residual activity from the plant’s roots on subsequent crops; this was termed “spent soil”. Second, the two crop plants were grown on soil mixed with *A. annua* dried leaves, this was termed “leaf-amended soil”. The germination and growth of the crops was measured and compared to the controls, which were grown on normal soil. Additionally, the beans were inoculated with *Bradyrhizobium* spp. and *Rhizobium* spp. (hereafter referred to simply as *Rhizobium*, as *Bradyrhizobium* does not normally infect *P. vulgaris*) to test the effects of *A. annua* on these beneficial symbiotic nitrogen-fixing bacteria. Photographs of each plant were taken at harvest, as were photographs of any relevant occurrences during the experiment.

**Corn germination and growth:**
Twenty 7cm x 7cm pots were filled with soil and watered from below, allowing the soil to absorb the water upwards to the surface. Gravimetric water content of the soil was found to be approximately 75%. Dried and pulverized *A. annua* leaves containing 1.8% AN by dry weight were added to ten of these pots at a concentration of 4g/m². Ten more pots were filled with spent soil and watered like the others. Then a corn seed was planted at a depth of 1cm in each pot and allowed to grow, without fertilization for three weeks after the first germination. Days to germination were recorded for each plant, and the height of each plant (from soil surface to tip of longest leaf) was measured every two days for a week after the first germination and every four days thereafter. At harvest, the height of the stem/longest leaf and the dry weight of the stem/leaves and roots were measured. This was repeated once more without the spent soil and with dried leaves at a concentration of 400g/m², a concentration used by Bohren et al. (2004). This set of dried *A. annua* leaves contained 1.3% AN by dry weight. This time the soil and the plants were watered from above until the soil was wet throughout, allowing the soil to settle.

**Bean germination and growth experiment:**
Twenty 11cm x 11cm pots were filled with soil and dried *A. annua* leaves were added to ten pots at a concentration of 4g/m². Ten more pots were filled with spent soil. These were then watered from below. The beans were then inserted into a plastic bag containing the *Rhizobium* inoculants and shaken thoroughly. A bean was then placed in each pot at a depth of 1cm and allowed to grow without fertilization for five weeks after the first bean germinated. Water was provided from underneath the pots. The time to germination was recorded for each plant and the height of each plant was measured (from soil surface to apical meristem) every two days for a week after the earliest germination and every four days thereafter. At harvest, the height of the stem was measured, the leaves were counted, and the nitrogen-fixing nodules were removed from the roots. The nodules and the rest of the plant were allowed to dry and the masses of the stems and leaves, roots (without nodules) and nodules were measured. This was repeated, but without the spent soil and with dried leaves added at a concentration of 400g/m² and with surface watering.

**Effect of fallow soil on bean germination delay:**
To determine the duration of the germination delay observed in beans, 40 6cm x 6cm pots were filled with soil amended with leaves at a concentration of 400g/m². This soil was watered from above every 2
to 3 days until the end of the experiment. Ten bean seeds were planted one week later and every week after that for three more weeks. The number of seeds that had germinated was recorded for each set of beans.

**Pure artemisinin germination:**
Professor Michael Timko’s laboratory (U VA) carried out an experiment in which *Striga hermonthica* seeds were exposed to pure AN. Fifteen replicates of 25mg of *Striga hermonthica* seeds were sterilized using 10% bleach and were preconditioned with water and GR 24, which mimics strigolactones and induces germination of *Striga* seeds. Seeds were incubated in Petri dishes at 30°C for 5 days. Then 10mg of AN were dissolved in 1ml of 70% ethanol and diluted in 250ml H2O (AN conc. 40ug/ml). Five replicates of *Striga* seeds were sprayed with 0.35% ethanol, 5 replicates of *Striga* seeds were sprayed with 40ug/ml AN and GR-24 was added to all replicates of *Striga* seeds. Seed germination was measured after 48 hours.

The effect of pure AN on the germination of both corn and beans was similarly tested. Thirty corn seeds and 30 bean seeds were placed in a 10% bleach solution for 5 minutes. The seeds were rinsed with sterile water and allowed to imbibe for an hour. Ten seeds of each crop were then dipped for 10 minutes in one of three solutions: 40µg/ml AN dissolved in 0.35% ethanol, 0.35% ethanol, and pure water. Seeds were then placed on moist autoclaved paper towels in 9cm diameter Petri dishes and allowed to germinate at 25°C in the dark. The number of seeds that germinated under each condition was recorded at 48 hours, 96 hours and 144 hours.

### 3.3 Soil Microbes
The microscopic ecosystem of the soil is complex and sensitive to relatively minute changes; this affects how nutrient cycling occurs in the soil and the availability and health of plant symbiotic microorganisms (Kennedy 1999). If the use of *A. annua* changes the way a crop plant grows, it may not be due to direct use of allelochemicals, but rather because *A. annua* changed the microbial composition of the soil, perhaps eliminating an important symbiotic partner or a harmful pest. The *Rhizobium* symbiont of beans is one indicator of *A. annua*’s effect on a key soil microbe. To determine the effect of *A. annua* on the bacterial community that can be extracted and cultured on agar, soil extracts were plated on TS (tryptic-soy) agar and allowed to grow. TS agar supports a wide variety of microbes (bioMerieux, Inc. 2009). The resulting colony count and morphology types (Appendix A) were used to determine whether *A. annua* growth had any effect on the community. Soil microbes are notoriously difficult to culture and only a small percentage (less than 1%) typically grow on plates (Kaeberlein et al. 2002), but the ones that do may still show general patterns, such as a change in density or colony diversity.

**Diluted samples from soil:**
The three types of soil were used: fresh soil, 400 g m⁻² leaf amended soil, and spent soil. A sample of each was brought to 75% gravimetric water content. A 1g sample was taken from each and diluted in 40ml filter-sterilized water. These solutions were then vacuum-filtered using No. 1 Whatman filter paper to remove larger particulates. The solutions were then brought to 100ml, a 1:100 or 10⁻² solution. Each was then serially diluted further with sterile water to produce 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions.
Microbial growth and characterization:
A 900µl aliquot of each dilution was plated on TS agar and grown at 25°C in the dark for a week. TS Agar consists of 15g Bacto tryptone (pancreatic digest of casein), 15g Bacto agar, 5g Bacto soytone (papain digest of soybean meal) and 5g NaCl, mixed with de-ionized water and autoclaved. The growth on each plate was monitored every 24 hours and, once sufficient growth had occurred, each plate was photographed. Each type of colony was qualitatively characterized and the number of colony-forming units (CFUs) of each type was determined. The colony characteristics are illustrated and defined in Appendix A (Leungh and Liu 2010).

3.4 Assays
Artemisinin
The artemisinin content of the dried leaves that were used in the experiments was analyzed by gas chromatography-mass spectrometry (GC-MS). The AN was extracted using a toluene extraction similar to that described by Towler and Weathers (2007). The dry tissue was homogenized and a 0.1g sample was taken and placed in a test tube. AN was extracted using 2ml of toluene in an ultrasonic water bath for 15min and the supernatant was filtered onto a new test tube. This extraction was repeated twice. The extract was then dried under a nitrogen stream in a 30°C water bath; 1ml of pentane was then added and the sample filtered again. A 100µl sample consisting of 10µl of the extract and 90µl of pentane was added to a vial containing 2.5µg of octadecane (ARCOS AC129296050) internal standard. This final sample (1µl) was analyzed in splitless mode with an HP-5MS column of dimensions 30m x 0.25mm x 0.25µm, in an Agilent 7890A GC system coupled with an Agilent 5975C MSD with triple-axis detector. The oven program is as follows: injection temperature was 250°C, detection temperature was 280°C, initial temperature was 120°C and ramped up to 300°C at 5°C/min, then held at 300°C for 15min. Ultra pure helium at a flow rate of 1ml/min was the carrier gas. Artemisinin was quantitated from a standard curve generated using authentic artemisinin (Sigma cat. No. 361593).

Inorganic Assays
Samples of fresh and spent MetroMix 360 were prepared and assayed for nitrate, phosphate and ammonia. A 10g sample of dried soil was dissolved in 100ml of hot de-ionized water and shaken vigorously for 10 minutes. Each sample was vacuum-filtered, as previously described. Some water was lost during filtration, so the volume of the solution was reconstituted to 100ml with de-ionized water. Inorganic nitrate, ammonium and phosphate content of the soil were determined using colorimetric assays.

Nitrate was measured using a Szechrome reagent consisting of 0.5g of Szechrome NAS (Polysciences, Inc. cat. No. 08762) dissolved in a 50:50solution of orthophosphoric acid (H₃PO₄): sulfuric acid 95-98% (H₂SO₄). This solution was allowed to stand a week before use. Then 137mg of NaNO₃ was dissolved in 100ml of HPLC grade water to produce 1mg NO₃/ml. This nitrate standard solution was diluted to triplicate 250µl samples of 10µg/ml, 20µg/ml, 40µg/ml and 60µg/ml solutions, to generate a standard curve against which the test samples were compared. A 2.5 ml aliquot of the Szechrome solution was added to each of the standards and to 250µl of each sample, mixed, and incubated for 45min. Another
solution consisting only of 250µl de-ionized water and 2.5ml reagent was prepared and used as a blank. The OD of each solution was measured at 570nm.

An ammonium stock solution was made using 88.9mg of NH₄NO₃ dissolved in 100ml HPLC grade water. The standard curve for this assay was created using triplicate 250µl samples of 8µg/ml, 16µg/ml, 24µg/ml, 32µg/ml and 40µg/ml NH₄ solutions. To each of these was added 0.5ml of phenol nitroprusside followed by 0.5ml alkaline hypochlorite. OD was measured at 570nm. Triplicate 250µl samples of the test solution were tested with the same reagent. The blank consisted of 250µl of de-ionized water and 0.5ml of each reagent.

For phosphate 9.44ml of H₂SO₄ were dissolved in 118ml of distilled water and 0.472g of ammonium molybdate (Sigma A7302-100G) was added. This solution was mixed with another solution consisting of 2.36g polyvinylpyrrolidone (Sigma P2307) dissolved in 118ml of water. Then, 0.96g of ferrous ammonium sulfate (NH₄)₂Fe(SO₄)₂ was added to the mixture. A phosphate stock solution consisting of 100mg of KH₂PO₄ dissolved in 100ml HPLC grade water was prepared. The standard curve for this assay was created using triplicate 100µl samples of 139.7µg/ml, 279.4µg/ml, 419µg/ml and 558.8µg/ml KH₂PO₄. The triplicate test samples each consisted of 100µl of the test solution. Each sample was treated with 3ml of the reagent and incubated for at least 10min before its OD was measured at 650nm.

### 3.5 Statistics
All plant experiments had at least 10 replicates. Data were averaged and analyzed using ANOVA and Tukey’s test, performed via the PASW Statistics 17 program by SPSS, Inc. where statistical significance corresponded to a p value ≤0.05.

### 4. Results

#### 4.1 A. annua’s Effect on Germination
Pure AN completely inhibited Striga germination and had no effect on the germination of either corn or beans (Table 3). All beans and 90% of corn exposed to AN and its ethanol control germinated within one day of each other, compared to 80% and 100% for the control for corn and the control beans, respectively. When soil was amended with 4g/m² A. annua leaves there was no dramatic difference in the germination rates of either beans or corn (Fig 5a-b).

**Table 3** Pure AN inhibits Striga but does not affect germination of corn and bean seeds. Corn and bean seeds exposed to 40µg/ml of artemisinin dissolved in 0.35% ethanol showed no decrease in germination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Striga (48hr)</th>
<th>Corn (48hr)</th>
<th>Beans (96hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure Water</td>
<td>75</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>0.35% EtOH</td>
<td>56</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>0.35% EtOH + 40µg/ml AN</td>
<td>0</td>
<td>90</td>
<td>100</td>
</tr>
</tbody>
</table>
Fig 5  Effects of *A. annua* on germination of bean and corn seeds. The germination rates of bean and corn seeds grown in normal potting mix (blue diamond), spent soil (green triangle), and potting mix amended with 4g/m² dried *A. annua* leaves (red square). The concentration of dried leaves was increased to 400g/m² and the watering method was altered from bottom to top watering for beans (c) and corn (d), respectively, in this experiment.
The percentage of bean seeds that germinated in 400g/m² leaf amended soil and the average time it took each to germinate. *Statistically significant at p ≤ 0.05.

<table>
<thead>
<tr>
<th>Week</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination (%)</td>
<td>90</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>Avg. Time to Germination</td>
<td>14.4*</td>
<td>7.6</td>
<td>8</td>
<td>5.4</td>
<td>5</td>
</tr>
</tbody>
</table>

Seeds that were planted in spent soil germinated the least: 70% of bean seeds and 80% of corn seeds (Fig 5a, b). All of these seeds germinated within one week. By the second week, 90% of the bean seeds had germinated and the control and 4g/m² leaf amended soil had 90% and 100% germination, respectively.

When the concentration of dried leaves in the soil was then increased a hundredfold (Fig 5c-d), there was no difference in the rate and level of corn germination between the control and the leaf-amended soil (Fig 5d). In contrast, there was a delay in germination of seven days for beans. After one week, 80% of the control bean seeds germinated, but only 20% of the seeds in the amended soil had germinated. By day 11, 90% of the control seeds germinated, while the seeds in the amended soil reached 90% germination at 14 days.

To determine if the inhibitory effect of *A. annua* in soil dissipated with time, soil was again amended with 400g/m² of dried *A. annua* leaves and a set of 10 beans were added every week for four weeks. The longer the *A. annua*-amended soil lay fallow, the better the germination (Table 4). After 3 weeks, germination time was equivalent to that of unamended soil (Fig 5).

### 4.2 *A. annua*’s Effect on Growth
The height of the corn and bean plants was not reduced by either spent soil or leaf amended soil (Fig 6) for either concentration of dried leaves. Despite significantly shorter plants for beans grown in 400g/m² amended soil (Fig 6c), this difference disappeared after two weeks and all bean plants reached approximately 30cm height within 21 days. The initial difference is likely the result of the delay in germination shown in Fig 5c. Although corn height seemed to decline when amended soil was increased from 4g/m² (Fig 3b) to 400g/m² (Fig 3d), the results are statistically insignificant.
Fig 6 The average height of bean and corn grown in control potting mix (blue diamond), potting mix that had been used to grow *A. annua* (green triangle), and potting mix amended with 4g/m² dried *A. annua* leaves (red square). The concentration of dried leaves is increased to 400g/m² and the watering method was altered from bottom to top watering for beans (c) and corn (d), respectively. Asterisks denote statistical significance for day 18 (a) between the *A. annua* spent soil and the others and on days 4, 6 and 8 (c) at p ≤ 0.05.
Fig 7 The final dry mass of the shoots and roots of bean and corn plants. White bars are control plants, gray bars are plants grown in A. annua spent soil and dark red bars are plants grown in leaf amended soil with leaf concentrations of 4g/m² (a, b) and 400g/m² (c, d). The asterisk denotes statistical significance at p ≤ 0.05.
Table 5 The ratio of dry shoot mass to root mass in plants that were watered from beneath and their total mass at harvest. Asterisk denotes statistical significance at p ≤ 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>A. annua - Spent Soil</th>
<th>Leaf-Amended Soil (4g/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot:Root Ratio</td>
<td>4.60</td>
<td>6.11 *</td>
<td>4.41</td>
</tr>
<tr>
<td>Final Mass</td>
<td>2.21</td>
<td>2.24</td>
<td>2.63</td>
</tr>
<tr>
<td><strong>Corn</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot:Root Ratio</td>
<td>3.86</td>
<td>5.62 *</td>
<td>4.45</td>
</tr>
<tr>
<td>Final Mass</td>
<td>0.420</td>
<td>0.608</td>
<td>0.469</td>
</tr>
</tbody>
</table>

Compared to growth in the control soil, the dry mass of bean plants was not significantly affected by growth in A. annua spent soil or leaf-amended soil (Fig 7a, c). Corn grown in the 400g/m² amended soil showed a significant increase in shoot biomass compared to controls (Fig. 7d); spent soil also showed a stimulatory effect (Fig. 7b). These data suggest that A. annua provides some benefit to growing corn. When the ratio of shoot to root mass in both crops was measured, it was, however, significantly greater for plants that were grown in A. annua spent soil than for either the control or the plants in leaf-amended soil at a concentration of 4g/m² (Table 5). This was surprising considering that the nutrient levels were somewhat depleted in the spent soil (Section 4.4). In contrast, the control corn plants that were watered from above showed a reversal in the ratio of shoot mass to root mass and a significantly lower mass than the 400g/m² amended soil (Table 6). A significant loss of shoot mass was observed in the control that was watered from above that was not present in the corn plants that grew in soil that was amended with 400g/m² dried leaves.

Table 6 The ratio of dry shoot mass to root mass in plants that were watered from above and their total mass at harvest. Asterisk denotes statistical significance at p ≤ 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leaf-Amended Soil (400g/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Mass</td>
<td>1.11</td>
<td>1.31</td>
</tr>
<tr>
<td>Shoot:Root Ratio</td>
<td>7.70</td>
<td>7.64</td>
</tr>
<tr>
<td><strong>Corn</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Mass</td>
<td>0.378</td>
<td>0.755</td>
</tr>
<tr>
<td>Shoot:Root Ratio</td>
<td>0.826*</td>
<td>5.01</td>
</tr>
</tbody>
</table>
4.3 Soil Microbes

*Rhizobium* nodules formed on all bean roots in the control plants, those grown in spent soil and those grown in $4g/m^2$ amended soil. The average nodule mass between these groups was not significantly different. In $400g/m^2$ leaf amended soil two of the plants did not have nodules in their roots. Although there appeared to be a decrease in the average nodule mass of the $400g/m^2$ group, the difference was not significant (Fig 8). The average ratio of nodule mass to shoot mass and to root mass was affected only in the bean plants that were grown in $400g/m^2$ dried leaf amended soil, dropping about 65% (Table 7).

![Graph a](image)

*Fig 8* The dry mass of the *Rhizobium* nodules found in (a) beans watered from underneath and (b) beans watered from above. Leaf amended soil had leaf concentrations of (a) $4g/m^2$ and (b) $400g/m^2$
Table 7 The dry mass of nodules extracted from the roots of bean plants grown in the control potting mix and in leaf amended soil and the ratio of nodule mass to shoot and root mass. Asterisk denotes statistical significance at $p \leq 0.05$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Leaf Amended Soil (400g/m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule Mass (g)</td>
<td>0.0775</td>
<td>0.0404</td>
</tr>
<tr>
<td>Nodule : Shoot Ratio</td>
<td>0.0805*</td>
<td>0.0298</td>
</tr>
<tr>
<td>Nodule : Root Ratio</td>
<td>0.597</td>
<td>0.193</td>
</tr>
</tbody>
</table>

The 100µg soil per ml ($10^{-4}$) extracts contained 27 colony types, several of which were too numerous to count (TNC). Those plates were not used for comparisons. Analysis of the 10µg soil per ml ($10^{-5}$) extracts yielded plates with distinct colonies that could be measured. The spent soil contained four fewer types of colonies and 131 fewer CFUs (Fig 9) than the control extracts. Out of a total combined 18 colony types and 641 CFUs, 6 colony types, yielding 598 CFUs were present in both types of soil extracts. Surprisingly, analysis of the leaf amended soil yielded no colonies. Microbial analysis of the leaf amended soil is currently being repeated to verify this result.
Fig 9 The diversity and abundance of colony-forming units extracted from control potting mix and A. annua spent potting mix. Some colony types were too numerous to count (TNC). The Venn diagram displays the distribution of the colonies from the 10µg/ml soil dilutions.

4.4 Assays
The phosphate and ammonium assays were not sensitive enough to detect either nutrient in the potting mix, all phosphate and ammonium levels were below the detectable range of each assay. On the other hand, the nitrate content in the control potting mix was 0.19mg/g, and in spent soil was 0.08mg/g. The AN concentrations for the 4g/m² and the 400g/m² leaves were 1.8% dry weight and 1.3% dry weight, respectively.
5. Discussion, Conclusion and Future Work

5.1 Discussion
The inhibitory effects of *A. annua* on bean germination are likely not due entirely to artemisinin *per se*; pure artemisinin failed to inhibit the germination of corn or beans. It was noted by Duke (1987) and seems likely in these experiments, that pure artemisinin affects small seeds much more than larger seeds. *Striga* is highly successful in part due to the small size of its seeds and pure artemisinin at a concentration of 40µg/ml, sprayed onto its seeds resulted in 100% inhibition. Dry leaf amendment at 4g/m² of 1.8% DW *A. annua* tissue at a depth of 2cm delivers an initial AN concentration of 3.6µg/cm³. Amendment at 400g/m² of 1.3% DW *A. annua* tissue delivers an initial AN concentration of 260µg/cm³. The higher leaf concentration likely delivered a higher amount of AN to the bean seeds than the pure AN test. However, the leaves also contain many other secondary metabolites as well as other artemisinic compounds and those may also play a role.

The nutrient depletion and alteration of microbial population observed in the *A. annua* amended soil did not seem to negatively affect the development of either crop. The significant increase in the mass of shoots and leaves of corn plants may be a result of stimulation by the roots of the potting mix microbiota. The spent soil had half as much nitrate as the control soil, but may have still had organically bound nitrogen and other nutrients sufficiently available. The corn in the 400g/m² leaf amended soil had significantly greater mass, which was not expected. Although the change in watering from below the plants to above the plants makes it challenging to compare data sets, the shoot mass of the 400g/m² amended soil corn plants is nearly equal to that of the shoots of the corn plants that were watered from below. In contrast, the average mass of the control plants is much lower, and the presence of *A. annua* leaves in the soil seem to have prevented corn from losing mass like the control plants did.

More importantly *Rhizobium* nodule formation was not affected by *A. annua* amended or spent soil, this agrees with recent field experiments performed by Morvillo et al. (2011), in which *Bradyrhizobium japonicum* nodule mass in soybean roots increased in the presence of *A. annua*. The nitrogen fixation provided by *Rhizobium* is critical to successful legume farming. In areas affected by *Striga* there is reported a high demand for edible legumes (Hooper et al. 2009). They also suggested the possibility of modifying beans, which can produce isoflavones similar to those in *D. uncinatum* that have been proposed as the chemicals that inhibit *Striga,* to produce more potent allelochemicals (Hooper et al. 2009). Legumes are also important for crop rotation, as they provide valuable fixed nitrogen to the soil. Although *A. annua* leaf debris does not seem to inhibit nodule formation, it may decrease nodule mass. On the other hand the decreased mass of the nodules in the leaf-amended soil was not statistically significant, but the nodule to shoot mass ratio was significantly decreased.

The lack of a significant change in dry mass for either corn or beans suggests that crop yields would not be negatively affected by either the effects of *A. annua* roots or leaf debris. This debris in the soil does not negatively impact corn, but rather to stimulate its growth. Furthermore, the leafy debris only results in about a one week delay in bean germination. This germination delay fades about a week after leaf...
deposition on the soil, and is completely eliminated after three weeks. Thus, at least for large seed crops like beans and corn, *A. annua* plant debris does not appear to pose a major problem for any farmer attempting to rotate crops with *A. annua*.

In contrast, however, Lydon et al. (1997) observed a significant decrease in corn germination and root mass in greenhouse soil amended with 474g/m² dry *A. annua* leaves, which disagrees with this study’s results. There are at least two possible explanations for this discrepancy. MetroMix 360 may somehow dampen allelopathic effects due to its composition, in which case a comparison of allelochemical activity between potting mix and natural soils would be useful. Alternatively the corn variety used by Lydon et al. (1997), which was not specified, may be different from the *Z. mays* F1 hybrid from Pinetree Garden used here, and possibly more susceptible to artemisinin. Clearly before implementation of *A. annua* into a rotation plan, native crops seeds should be tested for their susceptibility to artemisinin.

The short duration of the germination effect may also apply to any germination-related inhibitory effect that *A. annua* may have on *Striga*. If this is so, the usefulness of the medicinal plant in controlling the parasitic plant may also quickly fade. Careful management of *A. annua* as a *Striga* control method could be effective, but there is not enough information at this stage to formulate an agricultural plan. Furthermore, the interaction between live *A. annua* and *Striga* seeds may be different altogether, as artemisinin levels from the plant might not be high enough. If live *A. annua* does inhibit *Striga*, then it could be integrated into a system similar to Push-Pull. Indeed, Morvillo et al. (2011) showed that soybean yield did not decrease in the field in the presence of certain densities of *A. annua* allelopathy and competition. Other effects, such as the earthworm avoidance caused by artemisinin that was reported by Jessing et al. (2009) are also worth investigating with whole *A. annua* plants or soil amended with dried leaves.

5.2 Conclusions

*A. annua* leaf debris does not appear to be detrimental to corn or bean production. If it can also control *Striga* infestation, it could increase food security in Sub-Saharan Africa while also creating a greater supply of much-needed medicine. If *A. annua* does not inhibit *Striga* it will still be useful as a medicinal cash crop to increase artemisinin production. Further understanding of the plant’s effects on other important crops like sorghum and soybean will be needed. The effects of the plant on the soil microbiota, especially on *Rhizobium*, also do not appear to be detrimental to the long-term viability of the soil. Taken together, this study suggests that a more in-depth analysis of rotation of or intercropping of *A. annua* with food crops in Sub-Saharan Africa merits further investigation.

5.3 Future Work

*Striga* work must continue beyond pure AN inhibition, the effects of spent *A. annua* soil, leaf amended soil and live *A. annua* should be tested on *Striga*. Also, the effects of watering plants from above compared to watering from below are being investigated even as this project concludes. Although the effects of dried leaves on the soil microbiota appear to be inhibitory, that experiment is being repeated with 400g/m² dried leaves to verify that result. A concentration series of leaf amended soil should also be tested, and the changing populations of microbes in leaf-amended and spent soils over time should
also be measured. MetroMix 360 is potting mix and does not contain any natural soil, and as such will certainly have quite distinct properties from the vast variety of native soils throughout the world. It is important to compare the different microbial communities and AN kinetics in several types of agricultural soils with MetroMix 360.

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Image Credits:
Fig 2 (left): “Striga Weed Effect”. Western Seed Company. Accessed from:
www.westernseedcompany.com/images/Striga-weed-effect.jpg


Fig 4: “Push-Pull Technology”. International Centre of Insect Physiology and Ecology. www.push-pull.net/push_pull_works.htm

Appendix A

The following criteria were used to characterize each colony-forming unit (Leungh and Liu 2010):

**Form**: Circular, Irregular, Filamentous or Rhizoid

**Elevation**: Raised, Convex, Flat, Umbonate or Crateriform

**Margin**: Entire, Undulate, Filiform, Curled or Lobate

**Opacity**: Opaque, Translucent or Transparent

**Luster**: Dull, Medium, Shiny

**Size** (relative), from very small (<0.5mm) to very large (approx. 1.5cm)

**Color** (subjective)

A template for each form, elevation and margin type is provided in Fig 10. Other characteristics were also noted and each colony type was assigned an identifying number and mnemonic name for rapid identification.

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**Fig 10** The different types of colony morphologies as provided by Leungh and Liu (2010).