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Native Plant Allelopathy: A Potential Approach to Limit Invasive Grass Encroachment in Thorn Forest Restoration

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NATIVE PLANT ALLELOPATHY: A POTENTIAL APPROACH TO
LIMIT INVASIVE GRASS ENCROACHMENT IN
THORN FOREST RESTORATION

A Thesis

by

EMILY A. MULLINS

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

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LIMIT INVASIVE GRASS ENCROACHMENT IN
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December 2020

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ABSTRACT

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Less than 5% of the Tamaulipan thorn forest remains in the United States. For this reason, there have been many attempts at restoration of this ecosystem. Oftentimes these attempts are unsuccessful due to the high prevalence of invasive African grasses. In an effort to improve restoration efforts, native plant allelopathy has been examined for its ability to exclude one of these invasive grasses, guineagrass (*Megathyrsus maximus*). Native species were tested for allelopathy in bioassays using plant extracts. Species that exhibited allelopathy were evaluated in pot experiments for their ability to reduce beneficial fungal infection in guineagrass roots, grass stem mortality, and grass growth, and in the field as mulch for their ability to limit guineagrass cover and growth. Several potentially allelopathic species were identified. Mulch of *Zanthoxylum fagara* almost entirely excluded invasive grass growth and mulch from *Ehretia anacua* and *Vachellia schaffneri* reduced grass growth. This suggests that mulch from *Z. fagara* could be useful in restoration due to its ability to prevent guineagrass growth.

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CHAPTER I

INTRODUCTION

Tamaulipan Thorn Forest

The Tamaulipan thorn forest is highly diverse due to the location and sub-tropical climate which allow for convergence of both temperate and tropical species as well as serving as a migratory pathway for many animals. Approximately 1850 plant species can be found in this region, 1058 of which are not grasses, including over 170 species of woody plants (Judd et al., 2002; Smith et al., 2009; Richardson and King, 2011) and several widespread invasive, exotic grasses (Smith et al., 2009). The native flora consists of species that are drought tolerant (Foroughbakhch et al., 2001). These plants frequently exhibit adaptations to arid environments such as small leaves, deep tap roots, drought deciduousness, and thorns or spines (Reid et al., 1990; Cook et al., 2019; Alexander et al., 2015). Many of the species belong to the Fabaceae family, plants in this family are well known for their ability to sequester nitrogen via symbioses with nitrogen fixing bacteria and are also frequently allelopathic (Grove et al., 2017)

Plant community composition is determined by environmental variables such as water availability, soil type, and historical land use and management practices such as clearing for agriculture, grazing, and timber cutting (Reid et al., 1990). The Tamaulipan floodplain along the Rio Grande was once a continuous old growth mixed sabal palm and thorn forest but is now

highly fragmented by agriculture, urbanization, and changes to the water regime (Leslie, 2016). The only extant old growth sabal palm mixed forest is located in Cameron County, TX (Lonard and Judd, 2006). The sabal palm tree (*Sabal Mexicana Mart.*) is endangered in the United States (Lonard and Judd, 2006), though it is still prevalent in northeastern Mexico. Within plots of old growth mixed palm and thorn forests, the presence of invasive grass is very low despite the fact that adjacent plots, those that have experienced disturbance in the past, have a much higher prevalence of invasive grasses (personal observation at Sabal Palm Sanctuary, Southmost Preserve, and McAllen Nature Center). This suggests that community composition in old growth forests that may generate a protective shield against grass invasion.

The Tamaulipan thorn forest encompasses parts of the Gulf coast plain in northeastern Mexico and southeastern Texas. It is located in a transitional climactic zone between the tropical and the temperate climates (Leslie, 2016). Soil types vary widely throughout this region, from fine loamy sand to deep alluvial sediments composed of moderately fine clay (Adhikari and White, 2014). The climate of the tamaulipan thorn forest region is classed as humid subtropical and hot semi-arid. The Köppen-Geiger classification corresponds to Cfa, or humid subtropical (en.climate-data.org). Summers are hot and humid, average high temperatures are above 35°C for July and August. Winters are mild, the mean number of frost-free days per year is 330, though occasional severe frosts do occur (Adhikari and White, 2014). The majority of precipitation occurs during September and October, with an annual average rainfall of 680 mm (Judd et al, 2002). Rainfall is not uniform over the wet season; high volume rain events often supply a large portion of the yearly total (Leslie, 2016).

Anthropogenic Disturbances and Influence

Globally, habitat loss due to human development and the resulting fragmentation is the leading cause of native ecosystem degradation, accounting for a 22% reduction in the area that is covered by native ecosystems worldwide (Hoekstra et al., 2005). The Lower Rio Grande Valley (LRGV) of south Texas has been extensively impacted by habitat loss, much of this was cleared for grazing and agriculture, though rapid urbanization is becoming a large concern. Since 1940 the population of the LRGV has grown by over a million people and it is one of the fastest growing areas in the nation (Leslie, 2016). The building of the border fence along the border of the U.S. and Mexico will fragment additional land and will jeopardize the goal of habitat connectivity that has guided land management practices in south Texas since 1979 (Leslie, 2016). To date, more than 95% of the native Tamaulipan thorn forest in the LRGV has been lost and what is left remains in patches that are interspersed between urban and agricultural development (Sternberg, 2003). In Cameron County alone, over 74,000 hectares of native vegetation was lost to agricultural development between 1930 and 1980 (Lonard and Judd, 2006).

Grazing of livestock in this region has been occurring since the later part of the 1500s, contributing to degradation of the native ecosystem (Reid et al., 1990). The long history of grazing has led to the alteration of the native habitat from the state it was prior to European settlement. Cattle ranching and overgrazing led to widespread use of exotic African grasses as more productive forage and also as part of soil conservation strategies. The prevailing opinion at the time was that some ground cover was better than none (Alexander et al., 2015; Smith 2010), but several of the grasses used have become widespread invasive species in the region, notably buffel grass (*Pennisetum ciliare* (L.) Link), Kleberg bluestem (*Dichanthium annulatum* (Forsk.)

Stapf), and guineagrass (*Megathyrsus maximus*, [Jacq.] B.K. Simon & S.W.L. Jacobs (Poaceae), previously *Panicum maximum* and *Urochloa maxima* [Jacq.]) (Smith et al., 2009). The negative interactions between native species and invasive grasses pose the greatest limitation on the success of restoration efforts in the LRGV as the later are strong competitors and can be troublesome to control. Some methods of removal can promote further spread of invasive grass, such as clearing land with fire (Alexander et al., 2015).

Restoration in the Lower Rio Grande Valley

Forest restoration aims at recreating a functional forest habitat where it has disappeared or has been altered by some disturbance. Because there has been such a drastic loss of forested area in the LRGV it is important to restore as much habitat as possible in order to maintain ecosystem functionality and biodiversity into the future. An intact forest system provides many services such as nutrient cycling, soil stabilization, maintenance of the hydrological regime, increased biodiversity, and carbon sequestration (Cummings et al., 2012; Harris et al., 2006). Ideally, restoration would only require reintroducing native species based on either historical data or by mimicking an undisturbed reference site, but due to the dominance of invasive grasses and soil degradation, this is often not successful (Ammond et al., 2013; Cummings et al., 2012).

Restoration efforts in the LRGV began in the 1950s at Las Palomas Wildlife Management Area and were conducted by Texas Parks and Wildlife with the goal of improving hunting for white-winged doves. In the 1980s through the 1990s abandoned agricultural lands were acquired and seeded with woody species from the Fabaceae family that were fast growing, this was done with the intention of providing habitat for seed dispersing birds and animals that would facilitate secondary succession (Leslie, 2016; Sternberg, 2003). This method of restoring

abandoned cropland was not very successful due to low germination rates of the directly planted seeds, resulting in low species diversity in seeded sites, this method of restoration was abandoned in 1995 (Leslie, 2016; Stenberg, 2003). Beginning in 1995 most restoration efforts focused on planting a more diverse mixture of seedlings, typically 30-40 species; between 1995 and 2011 over 3 million seedlings were planted in restoration efforts. (Leslie, 2016)

Seedling survival and establishment is still a challenge and several potential solutions have been explored. Vertebrate herbivores are one serious obstacle to the survival of seedlings; experiments using shelter tubes and exclosures have shown that they promote growth of tree seedlings more than herbicide application alone (Dick et al., 2016). Because most of the sites slated for restoration are located on old agricultural fields, the soil is often degraded and has a seed bank containing invasive and weedy species, and constitute an open niche. In the LRGV and elsewhere, many disturbed habitats are aggressively populated with invasive C4 grasses that negatively impact natural succession and reduce the success of restoration efforts (Cummings et al., 2012). Controlling invasive species is required for seedling survival; current methods of achieving this include plowing or disking the soil and the application of herbicides before and after plantings (Alexander et al, 2015). One potential solution to aid restoration success is to plant diverse mixes of native seeds to compete with and suppress the regrowth of invasive grasses (Smith et al., 2009). This could be enhanced by carefully choosing species that produce allelopathic chemicals that inhibit the germination, growth, and development of non-native species; as well as including early successional grasses, and large stature forbes.

Allelopathy Background

Allelopathy can be considered a type of communication between plants, in which chemical compounds released from one plant influences the growth and development of another plant. The concept of allelopathy was first recorded in 300 BCE by Theophrastus, who observed that chickpeas “exhausted” the soil and destroyed weeds. The term allelopathy itself was coined in 1937 by Hans Molisch, a plant physiologist from Austria. Proving allelopathy to be the cause of negative interactions between plants has been quite challenging as it is difficult to isolate the impacts of allelopathy from other ways that neighboring plants can influence one another such as competition for light, nutrients and water (Inderjit et al., 2009; Cipollini et al., 2012). Often, strongly allelopathic plants will exhibit a halo consisting of bare soil surrounding the plant in which no other plants grow. Williamson (1990) argues that allelopathy has been charged with a greater burden of proof than other types of plant-plant interactions, such as resource competition, and for this reason it is important to plan experiments in such a way that the allelopathic component is separated from other factors.

There are three primary ways that allelochemicals can be released into the environment: as volatiles from leaves, exudates from roots, or by leaching from the canopy and fallen litter (Mello and Oliveria, 2016). Though allelopathy can have stimulatory effects on neighboring plants (Cummings et al., 2012), most often the noted effects are negative. These chemicals can lead to many changes in the physiology of the recipient plant such as increased permeability of the cell membrane, inhibition of nutrient absorption, reduced rates of germination, inhibition of root elongation, changes in cell structure, altered enzyme functionality, and impaired photosynthetic capabilities (Li et al., 2010). Often conditions that stress the plant can lead to an

increased production of allelochemicals and an amplification of the effects of allelopathy (Cummings et al. 2012) including impacts on microbial symbionts.

Mycorrhizal fungi form symbiosis with plants through an association with the roots of the plant. The fungi scavenge the soil for water and nutrients that are then made available to the plant in return for carbohydrates derived from photosynthesis (Taiz et al., 2018). It is estimated that 90% of terrestrial plants form these symbioses (Hale and Kalisz, 2012), and the majority, about 80%, are formed with arbuscular mycorrhizae (Taiz et a., 2018). Arbuscular mycorrhizal fungi (AMF) is a type of mycorrhizal fungi in which the hyphae penetrate the cortical cells of the root (Taiz et al., 2018). It is likely that these associations are what facilitated the colonization of land by plants, and they are still incredibly important to the success of many plants today (Smith et al., 2008).

AMF belong to the phylum Glomeromycota and are obligate symbionts (Smith et al., 2008; Taiz et al., 2018). AMF form structures known as arbuscules within the root cells of the plant. These arbuscules are not visible to the naked eye; in order to visualize these structures, the roots must be stained and examined microscopically (Smith et al., 2008). Outside the root cells the AMF form hyphae that are highly branched and extend past the zone of nutrient depletion, allowing the plant access to less mobile nutrients such as phosphates (Taiz et al., 2018).

Invasive plants can change the microbial community composition in the soil by releasing allelochemicals that inhibit the growth of native plants that depend on mycorrhizal symbioses (Cipollini et al., 2012). If the invasive species gain a competitive advantage, the microbial community may change. This change happens because the invasive species may not form mutualistic symbioses with fungi, may be less reliant on fungal mutualisms, or may form mutualisms with species that vary from those of the native plants. Over time, following

colonization by an exotic invasive, shifts in the microbial community occur and begin to more closely resemble those in the original range of the invasive species. The invasive species cause changes to the soil microbiome by changing the food source and through allelopathy (Grove et al., 2017). The changing food source impacts the soil community by providing different host plants for plant-fungal mutualisms. Many species are not generalists and can only form mutualisms with a subset of the plant community so when an exotic species invades the native flora, there are fewer appropriate plants to colonize. The invasive plant also provides a host for microbial species with which it co-evolved, thus allowing for a shift in the soil community to occur. Allelopathy can also contribute to this shift in the mycorrhizal community through direct negative effects on AMF (Callaway et al., 2008).

Several invasive plant species have been identified as allelopathic that act through inhibition of AMF, perhaps lending to their ability to be successful invaders. Some plants, for example many brassica species, do not form symbioses with mycorrhizal fungi allowing plants from this family to inhibit mycorrhizae without harming themselves. Another case is that of *Alliaria petiolate*, aqueous leaf extracts from this plant inhibit spore germination of AMF, the effect of this plant is much stronger on AMF species that do not share an evolutionary history with *A. petiolate*. Inhibition of spore germination led to declines in the growth of native trees in the invaded regions and Cipollini et al. (2012) suggest that this is caused by the change in the structure of the microbial community.

Utilization of Allelopathy in Agriculture and Restoration

Allelopathy has been used in agriculture for several decades and many common crops are known allelopaths (Arif et al., 2015; Putnam and Dukes, 1979; Farooq et al., 2011.). Crop

rotation with allelopathic crops is one of the most common uses, this results in the inhibition of weed germination and tends to produce residual positive effects on the crops that follow (Bajwa et al., 2015). Some examples of allelopathic crop rotations include *Sorghum* sp., *Brassica* sp., and Alfalfa (*Medicago sativa* L.) (Kohli et al., 2006). One of the main reasons allelopathy is successful in reducing agricultural weeds is that many of these plants are either annual or biennial, occurring in recently tilled fields, and allelopathy tends to have the largest impact during the initial germination and growth stages (Bajwa, 2014). Teasdale et al. (2012) found that growth of weeds, in a field that had been previously planted with rye, was inhibited for 4 weeks after the rye had been incorporated into the soil. This was despite the short persistence time of the allelochemicals, which was estimated to be two weeks. This extended effect could be attributed to changes in the soil microbial community that persist after the chemical itself has degraded.

Though allelopathy has been studied for its usefulness in agriculture for decades, it has rarely been examined for its applicability in forest restoration. Woody plants' ability to produce allelochemicals is well known; the American black walnut (*Juglans nigra* L.) has been well studied and its active allelopathic chemical, juglone, has been identified in other species within the walnut family (Achatz et al., 2014). Because restoration sites tend to be tilled before planting to remove unwanted and weedy species, they have similar conditions to agricultural fields, this suggests that there are potential ways to use allelopathy in restoration in much the same way that it is used in agriculture.

The “novel weapons” hypothesis, proposed by Callaway and Ridenour (2004) suggests that some plant species are able to become invasive because they produce biochemicals that are inhibitory to plants and soil biota that did not coevolve with the plant, and that these same

biochemicals have little to no inhibitory effect on other species in their native range. Support for the novel weapons hypothesis has been observed in multiple studies, one such example examined the effect of an allelochemical produced by *Centaurea maculosa* L. ssp. *micranthos* (Gugler) Hayek, which is a forb native to Europe and Asia that is invasive in western North America. Laboratory experiments showed strong negative effects on North American species but only weak impacts on species that evolved alongside *C. maculosa* (Thorpe et al., 2009). Another study found that the impact of allelochemicals appeared to be dependent on where geographically the soil and its attendant microbes were sourced. Soils from regions in Europe where *Alliaria petiolata* is native and regions in North America, where it is invasive were compared in relation to the growth of plants in soils that had been cultured with *A. petiolata*. This resulted in reduced biomass of plants grown in N. American soils and no negative effect on plant biomass in soils from Europe, or in the case of soil from Spain, an increase in plant biomass was observed (Callaway et al., 2008).

Cummings et al. (2012) proposed the “homeland security” hypothesis which suggests that a similar effect to that seen in the novel weapons hypothesis could be at play in intact ecosystems, allowing them to resist invasion because novel species will be intolerant of the allelochemicals produced by the native plants. Cummings et al. (2012) point out that many plants in the Fabaceae family produce allelopathic alkaloids and they found that there was a lower presence of *Saccharum spontaneum* L., an invasive C4 grass, under legume species than under deep shade and species from other families, which suggests that allelochemicals from the legumes are suppressing the growth of the grass. They performed an experiment placing leaves on the surface of containers with growing *S. spontaneum*. After surface watering for 7 weeks the researchers found that leaves from legume species reduced the survival of *Saccharum*

significantly though there were differences between species. Further research examining legume and non-legume species concluded that leaf litter of some species can benefit the grass, calling attention to the necessity for screening prior to implementation. There are many species from the Fabaceae family in the Tamaulipan thorn forest and it is possible that allelopathy could be what allows undisturbed thorn forest to resist invasion. The presence of allelopathic species in this plant community and possibilities for uses within a restoration context should be further explored.

Studies to determine allelopathic species usually begin by running petri dish bioassays on lettuce seedlings using aqueous leachates of suspected allelopathic species. Shiraishi et al., (2002) used lettuce seeds to screen 71 ground cover plants for allelopathy prior to testing against target weed species. The ecological relevance of bioassay experiments is often called into question due to the use of unrealistic leachate concentrations and the use of sensitive target species such as lettuce. It is also important to note that just about any species can produce substances toxic to some other species at high enough concentrations (Inderjit and Calloway, 2003). For this reason, field studies are indispensable in confirming allelopathy and, in particular, the utility of allelopathic mulch as a means to reduce the presence of invasive grasses in restoration plots.

Hypotheses and Objectives

Invasive grasses are a major limiting factor in thorn forest restoration in south Texas. Allelopathy is often used to reduce the presence of weeds in agriculture and could serve the same function in restoration. The objective of this study was to determine which native plant species exhibit allelopathy in laboratory bioassays, pot experiments, and are effective in the field. Four

hypothesis are being tested: 1) Some plants native to the Tamaulipan thorn forest exhibit allelopathic effects that impact germination and seedling growth in laboratory bioassay experiments; 2) ground leaf material of allelopathic native plants will reduce the growth and survival of guineagrass when grown in pots; 3) allelochemicals present in native plants will reduce the presence of AMF in guineagrass roots thereby reducing the fitness of the grass; 4) the allelochemicals present in native species will limit the growth and cover of guineagrass in anthropogenically disturbed sites. To test these hypotheses, a series of laboratory and field experiments were performed.

CHAPTER II

METHODS

Study Area

The study area is located at The Nature Conservancy's Southmost Preserve near Brownsville, Texas. The preserve is 4.1 km² and contains one of the last old growth Mexican sabal palm and thorn forest mixed stands remaining in the United States. The study took place in a 27-hectare portion of the preserve that had been an organic grapefruit orchard. The orchard was cleared in 2014 and left fallow. The field is currently dominated by guineagrass. The region has a semi-arid and subtropical climate. The summers are hot with mild winters. Most of the precipitation in the region falls during the months of September and October, with an average yearly rainfall of 680 mm (Judd et al, 2002). Soils consist of 61% silt, 28% sand, and 11% clay (NRCS Web Soil Survey)

Species Selection and Aqueous Extract Preparation

Potentially allelopathic species were selected based on field observations in areas with slight disturbance. Criteria used for selection were apparent reduced understory growth and exclusion of grasses (Fig. 1). Additional test species were suggested by individuals familiar with the local flora. Approximately 250 g of fresh leaf material was collected from each of the 11 selected species during May, 2019. Care was taken to ensure that there were no stems, fruit, or flower parts in the materials collected. On the day of collection, the fresh leaf material was

placed into brown paper bags with holes for increased air circulation. These bags were placed in a drying oven set at 40°C until the material was thoroughly dry. The dry material was ground to a uniform powder and stored at room temperature in 1000mL Nalgene™ Lab Quality Amber bottles. To make the aqueous extracts, 20 g of ground leaf material was added to 450 mL of deionized water (DI) in beakers covered with aluminum foil to reduce exposure to light. A stir bar was added to each beaker and they were set on stir plates at room temperature for 24 hours. After 24 hours, the mixture was strained through a fine plastic mesh and placed in amber Nalgene bottles. The concentration of the initial solution was 40 g of dry ground leaf matter per liter of solution (100% concentration). A 66% concentration was made by mixing 66mL of the 40g/L solution with 34mL DI water. A 33% dilution was made by mixing 20mL of the 66% solution with 20mL DI water (Overholt et al., 2012; Khasabulli et al., 2018). The extracts were protected from light in amber bottles or wrapped in aluminum foil and were stored in the refrigerator to be used within one week. Conductance and pH of all extracts and dilutions were measured using Hach Hq40d Portable Multi Meter to determine if either of these factors affect germination or initial seedling elongation (Medina-Villar et al., 2017).

Laboratory bioassays using the aqueous extracts were performed to determine if the selected native species exhibit allelopathic potential in a laboratory setting. Each treatment was compared to the DI water only control and to the other treatments. Species that appeared to have a greater impact than the others were then tested in the field and pot experiments.



Fig. 1. A halo of bare ground around the base of *Z. fagara* in an area otherwise invaded by guineagrass.

Laboratory Bioassay

Seed Germination

A petri dish bioassay was performed on lettuce seeds to test which native species impact seed germination and initial growth of lettuce seedlings, negative impacts could indicate allelopathy. Three layers of filter paper were used in the petri dishes, two below the seeds, one above. There were 34 treatments in total: 11 species each with 3 concentrations (100, 66, and 33 percent) with DI water used as the control. Four replicates (petri dishes) of each treatment were performed for a total of 136 petri dishes. Twenty lettuce seeds were evenly placed in each petri dish, lettuce seeds were used because they have a high germination percentage, they germinate quickly, and are sensitive to allelopathic compounds. For these reasons, lettuce is considered to be a model species for use in surveying for allelopathic potential (Hernandez-Aro et al., 2015).

Aqueous extracts (2.5 mL) were added to each plate. Due to the rapid germination of lettuce seeds, the addition of the extracts was staggered, with one treatment started every 10

minutes, this was done to account for the time it takes to count and measure the seeds to ensure that each dish could be recorded every 24 h precisely. The seeds were kept at room temperature (24°C) underneath a plant light with a 12 h photoperiod. Seeds were recorded as having germinated when the radicle reached 3mm in length. Radicles were measured with a clear plastic ruler. Seeds were discarded once they had been counted as having germinated to eliminate the chance of counting a seed more than once. The test was considered finished when no seeds had germinated for three consecutive days (Mello and Oliveira, 2016).

Seedling Length

Lettuce seeds were allowed to germinate and seedlings allowed to grow undisturbed at room temperature and a 12 h photoperiod for 5 days. The seeds were placed in petri dishes with two layers of filter paper below and one above. Fifteen seeds per treatment were arranged in three rows of five within the petri dish, to reduce the chances of radicles becoming entangled. The seeds were watered with the aqueous extract when the filter paper began to dry out. At the end of the 5-day growth period, seedlings were straightened along a ruler and the length of the entire seedling (radicle and hypocotyl) was recorded. Table 1 shows each thorn forest species that was tested in the bioassay experiments and which metrics may exhibit allelopathy. Most treatments varied significantly from the control so to select species for further testing the treatments were compared to one another. Selection was based on impacts seen at the lowest extract concentration, those species that had a greater impact on the tested metrics than the average of all treatments were considered to exhibit allelopathy in that metric. Species that exhibited allelopathy in three or more metrics were further examined in the pot and mulch experiments.

Pot Experiment

Guineagrass Growth

Guineagrass was grown in pots to examine how leaf litter from allelopathic native plants impacts growth and survival of the grass. Guineagrass plugs or clones of equal size were taken from a single location in a guineagrass-invaded plot at TNC Southmost Preserve. The individual clones were transferred to one-gallon pots filled with soil from the location where the grass was collected. The plugs were allowed to grow for 5 weeks in the pots prior to the beginning of the experiment. Above ground plant matter was trimmed to a uniform height of 10 cm at the beginning of the experiment and all but 10 stems were removed. Four grams of dried and ground leaf litter from the potentially allelopathic species were placed on top of the soil in the pots (Cummings et al., 2012). The plants were surface watered as needed to allow the leaf litter to release compounds into the soil. The plants were fertilized at week 4 of the experiment with a 15-30-15 nitrogen-phosphorus-potassium fertilizer in aqueous solution. A completely randomized design with six replicates was used. Treatments included dry ground leaf material from *A. trisulcatum*, *E. ebano*, *M. drummondii*, *V. schaffneri*, *Z. Fagara*, and *R. humilis*, *T. inula* as well as a control treatment with nothing added.

Maximum height was recorded at 21 days and 49 days after leaf litter was added to the pots. The above ground biomass was removed from each pot, dried for 7 days at 40° and weighed. At the completion of the 7-week growth period the number of dead and surviving stems of grass were counted. Each pot had 10 stems at the beginning of the experiment. For any pot in which all ten stems could not be accounted for, the number of surviving stems was subtracted from ten in order to determine mortality.

Guineagrass AMF Infection

At the end of the 7-week growth period, samples of fine root hairs were taken from each pot of guinea grass. The roots were washed in a small sieve in order to remove soil then cut into 1.5 cm pieces. To clear the roots, samples were immersed in 10% KOH in 1.5 mL micro-centrifuge tubes for 2 hours in an 80°C water bath, following this samples were rinsed twice with water. Samples were then be immersed in Trypan blue/lactoglycerol for 24 hours then rinsed twice with tap water. The root fragments from each plant were lain horizontally on a slide, covered with a cover slip and sealed with clear nail polish (McGonigle et al. 1990). Overall, AMF infection in the roots was too low to determine the percent infection. Roots were only evaluated for AMF presence or absence.

Field Experiment

Mulch Plots

Mulch was made from four woody thorn forest species using a commercial wood chipper. Mulch was made of live leaves, twigs, and stems and was used instead of leaf litter due to the volume required in restoration efforts. Mulch also persists longer in the field than leaf litter does due to the lignin content in the wood. Three of the species selected for this experiment were those that appeared to have the strongest allelopathic effect in the seed bioassay and in the seedling growth experiment and one was used as the control (i.e. non allelopathic). The allelopathic species were *E. ebano*, *V. schaffneri*, and *Z. fagara*. whereas *E. anacua* was used as the non-allelopathic control, as this species appeared to have little to no negative impact on

germination and initial seedling growth. Additional plots with no mulch were set up as a mulch-free control. The experimental design was a completely randomized design consisting of five treatments and with five replicates. This experiment took place at The Nature Conservancy's Southmost Preserve. The plots were established on October 11th, 2019 in a field that was completely invaded by *M. maximus* (Fig. 2). Prior to the establishment of the plots, the field was disked under two times. It was assumed that there was a large seed bank of *M. maximus* in the soil to allow for a quick recolonization if not impeded.



Fig. 2. The mulch plots in the newly cleared field at the beginning of the experiment (October 11, 2019).

The plots were 1.0 m², the area to be measured sits in the center of the square with a 25 cm buffer on all sides, resulting in 50 x 50 cm area in the center of the plot to be evaluated. The plots were separated from one another by a minimum of 1 m. Thirty liters of mulch were spread evenly over each plot, resulting in a mulch layer approximately 3 cm thick. Percent cover of guineagrass and other species present were recorded monthly from November, 2019 to September 2020. Percent cover data is missing for the month of December and all data is missing for the months of April and June due to the coronavirus pandemic resulting in site access restrictions. Percent cover was determined using images analyzed with SamplePoint software (SamplePoint v1.59). At the end of this period, the aboveground biomass of guineagrass was

collected. The fresh mass was recorded in the field. A representative subsample from each treatment was dried and weighed. The fresh mass to dry mass ratio of the subsamples were used to calculate the dry mass of guineagrass in each plot.

Seed Plots

Plots were set up at The Nature Conservancy's Southmost preserve in the same manner as the mulch plot test. 1.0 m² plots were established in February, 2020 in a recently disked area that once was a grapefruit grove and is now dominated by guineagrass. These plots were seeded with the native herbaceous species that exhibited allelopathy in the bioassay experiments, *R. humilis*, *M. dromundii*, and *A. trisulcatum*, as well as *T. inula* which was suggested by A. McDonald. The seeds were gently raked into to soil to ensure soil contact and to reduce removal by wind. Within the plots, a 0.5 m² section was marked out in the center, leaving a 25 cm wide buffer. The plots were checked for seedling germination in March, May, July, and August. None of the experimental species germinated during this period. No data was collected from this experiment.

Data Analysis

In the bioassay experiments, total germinability (G, %), seed germination index (SGI), and mean germination time (MGT) in days were calculated using the following formulas. Germinability describes the percent of seeds that germinated, SGI and MGT are two different approaches to evaluate the speed and synchronicity of the germination process.

$$G = \frac{\text{Total germinated seeds}}{\text{Total seeds}}$$

$S_{GI} = \left[N_1 + \frac{N_2}{2} + \frac{N_3}{3} + \dots + \frac{N_n}{n} \right]$ Where N_1, N_2, N_3, N_n , are the proportions of seeds germinated at days 1, 2, 3, n, respectively, from the beginning of the bioassay.

$MGT = \frac{\sum_{i=1}^k n_i t_i}{\sum_{i=1}^k n_i}$ Where t_i is the time from the start of the experiment to the i th observation in

days, n is the number of seeds germinated on the i th time, and k is the last day of germination.

All statistics were run in R, version 3.5.1. The bioassay data did not meet the assumptions of normality even following log transformation, for this reason non-parametric tests were used to assess differences between the treatments. The Kruskal-Wallis rank sum test, and pairwise Wilcoxon rank sum test were used to assess effect of extract/dilution on: G, MGT, SGI, and seedling length. A Pearson correlation was performed between extract pH and MGT, SGI, and seedling length as well as between SPC and MGT, SGI, and seedling length. The correlation between pH and SCP and G was not performed because of the high rate of germination across treatments.

The data from the pot experiments and field mulch experiments met the assumptions of normality in some cases, for those metrics an ANOVA was run followed by Dunnett's test or t-tests, for those that did not meet the assumptions of normality, Kruskal-Wallis rank sum test and Pairwise Wilcoxon rank sum test were run to determine significant differences between the control and the treatments. In the pot experiment the parameters examined were: grass height, and aboveground biomass. The parameters assessed in the field mulch experiments were percent cover of guineagrass and standing aboveground guineagrass biomass. AMF infection in the guineagrass roots was too low to analyze statistically.

CHAPTER III

RESULTS

Laboratory Bioassay

Seed Germination

After six days nearly all treatments had 97.5% germination or higher. The exceptions were *E. ebano* 100% concentration (84% germination), *R. humilis* 100% concentration (5% germination), and *R. humilis* 66% concentration (80% germination). Differences in germination were observed between treatments, particularly with the highest extract concentration (Fig. 3). While most treatments did reach nearly 100% germination, there were differences in when germination began. Extracts made from *R. humilis* differed the most from the others particularly the 100% concentration. In this treatment seeds did not begin germinating until day 4 and on average only 5% of seeds germinated.

The SGI for all treatments was lower than the control indicating an increase in the time to germination. For all species the lowest extract concentration (33%) had the highest SGI, and the highest concentration had the lowest (Fig. 4). *R. humilis* had the lowest SGI across all concentrations with values of 0.94, 24, and 48. *E. ebano* had the second lowest SGI across all concentrations with values of 22, 33, and 50. For all treatments, the mean SGI for 100% concentration SGI was 39.4, for 66% concentration mean SGI was 48.8, and for 33%

concentration the mean SGI was 58. SGI for the control was 95 (Fig. 4).

The Kruskal-Wallis p-value for SGI was less than 0.005 indicating that there were significant differences between the treatments. To determine which treatments differed significantly from the control, a pairwise Wilcoxon rank sum test was used. This test indicated that all treatments were significantly different from the control for this germination metric. Because all species were selected for being apparently allelopathic in the field, this was not unexpected. To determine which species showed the highest allelopathic potential, as compared to the others, the mean SGI was calculated and plotted against the box plots for each treatment at the 33% concentration. All species with an SGI below the mean were considered for further testing in the pot and field experiments.

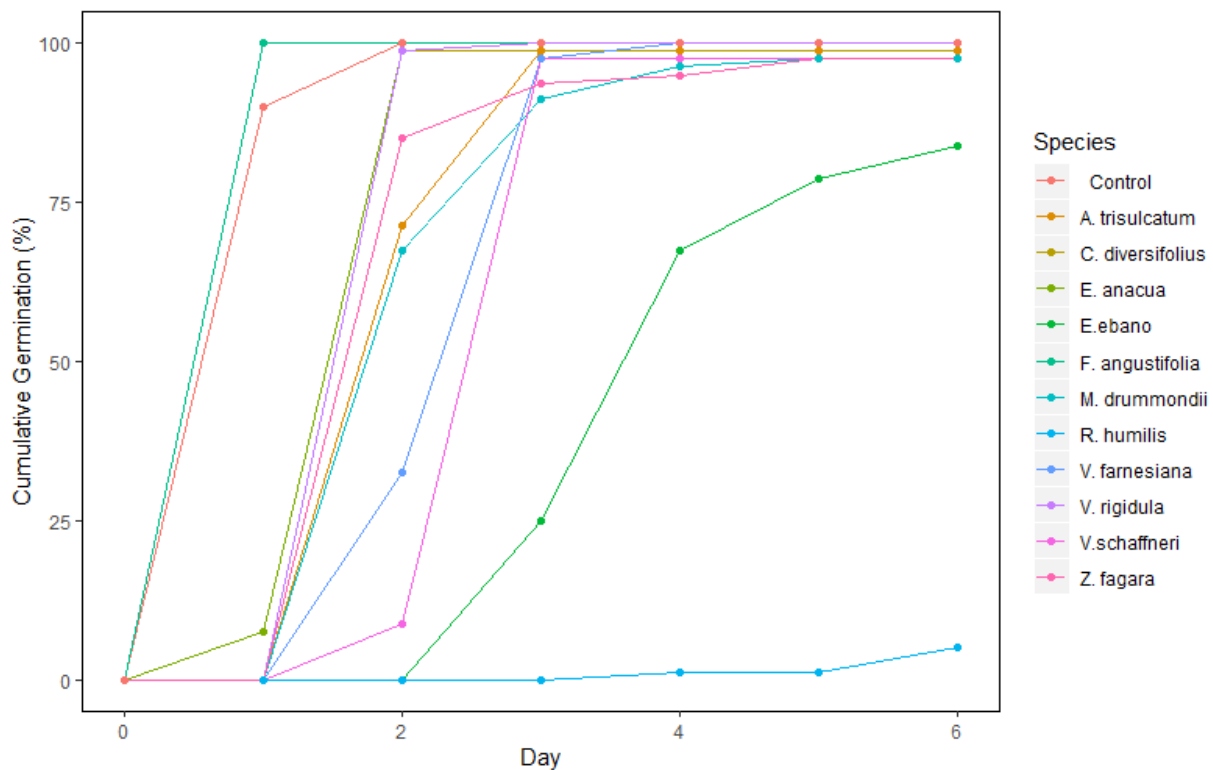


Fig. 3. Percent germination over time of lettuce seeds treated with the 100% concentration aqueous extracts from leaves of thorn forest species. The two species that inhibited germination the most were *R. humilis* and *E. ebano*.

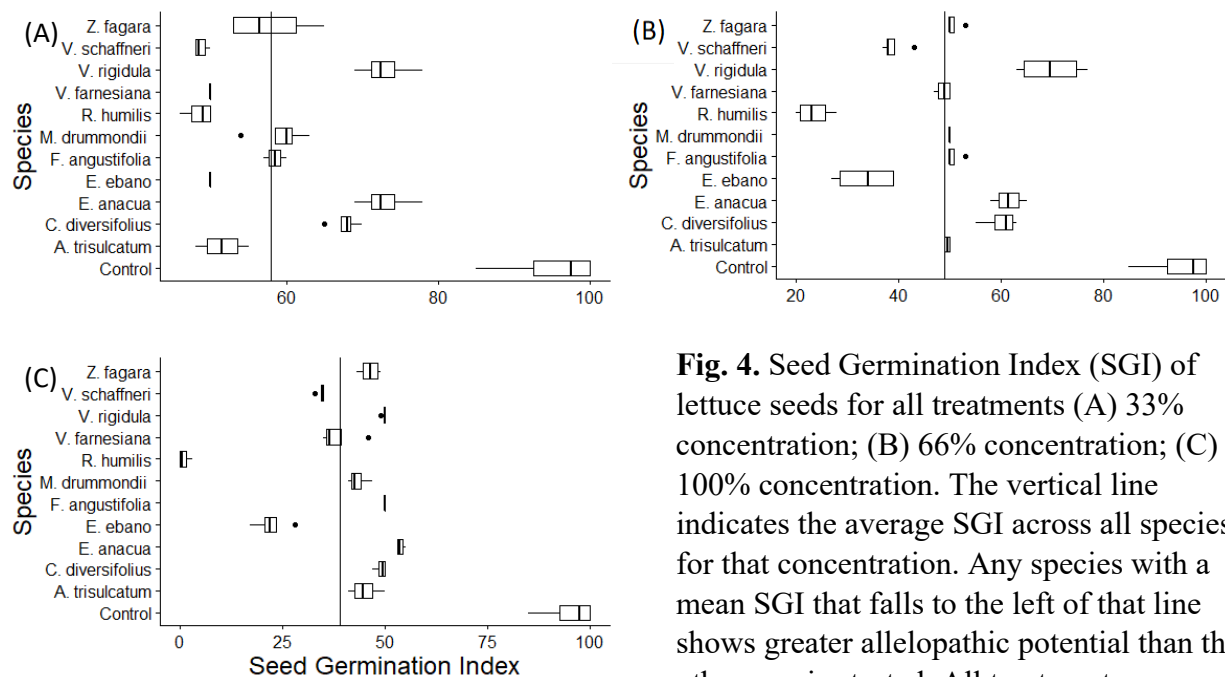


Fig. 4. Seed Germination Index (SGI) of lettuce seeds for all treatments (A) 33% concentration; (B) 66% concentration; (C) 100% concentration. The vertical line indicates the average SGI across all species for that concentration. Any species with a mean SGI that falls to the left of that line shows greater allelopathic potential than the other species tested. All treatments were significantly different from the control

The MGT of all treatments were significantly different from the control. (Fig. 5). The highest MGT was obtained with *R. humilis* and *E. ebano* extracts. For the 66% and 33% concentrations of *R. humilis*, the MGT were 3.6 and 2.1 days respectively. MGT of *E. ebano* at 33%, 66%, and 100% concentrations were 3.98, 3.19, and 2.00 days, respectively. MGT could not be calculated for *R. humilis* at the 100% concentration because no seeds germinated in two of the replicates resulting in a zero in the denominator. Across all species, the mean MGT for 100% concentration was 2.59, for 66% concentration MGT was 2.24, and for 33% concentration MGT was 1.83 days. MGT for the control treatment was 1.10 days. As in the SGI metric all species were selected because they allelopathy in the field thus all treatments differing significantly from the control was expected. To determine which species showed greater allelopathic potential, as compared to the others, the average MGT of each concentration was plotted against the box plots

for each species. All species at the 33% concentration with an MGT above the mean were considered to be more allelopathic than the other species and were utilized in the pot and field experiments.

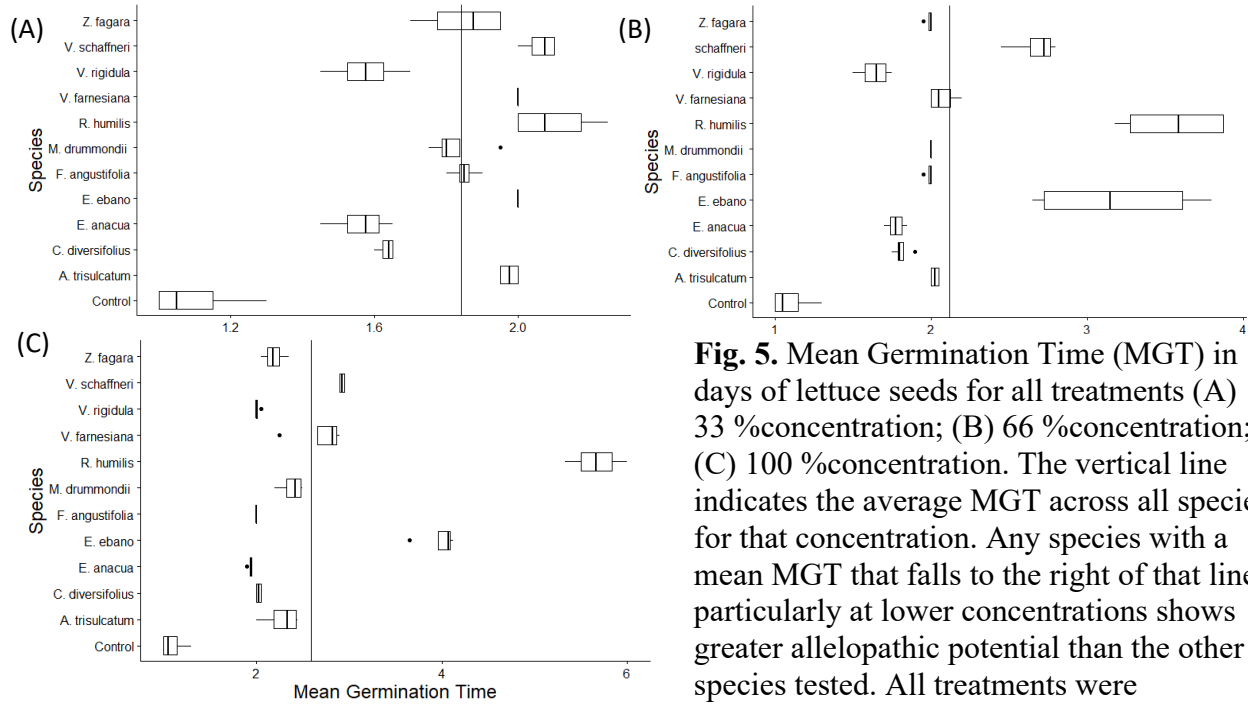


Fig. 5. Mean Germination Time (MGT) in days of lettuce seeds for all treatments (A) 33 %concentration; (B) 66 %concentration; (C) 100 %concentration. The vertical line indicates the average MGT across all species for that concentration. Any species with a mean MGT that falls to the right of that line, particularly at lower concentrations shows greater allelopathic potential than the other species tested. All treatments were significantly different from the control.

Seedling Length

Seedling length varied from 0 mm to 63 mm at the completion of the experiment. The average seedling length for the 33% concentration treatments was 51 mm, for 66% concentration the mean was 45 mm, and for the 100% concentration the mean was 34 mm. At the highest concentration, all treatments except *C. diversifolius* had a significantly lower mean length than the control. At lower concentrations, several treatments had a longer mean seedling length than the control, only *E. anacua* 33% concentration treatment was significantly longer than the control (Fig. 6). *E. ebano*, *R. humilis*, and *V. schaffneri* had the shortest mean length across all

concentrations. Table 1 shows which metrics each species exhibited allelopathy at the lowest concentration, summarizing the results shown in the box plots.

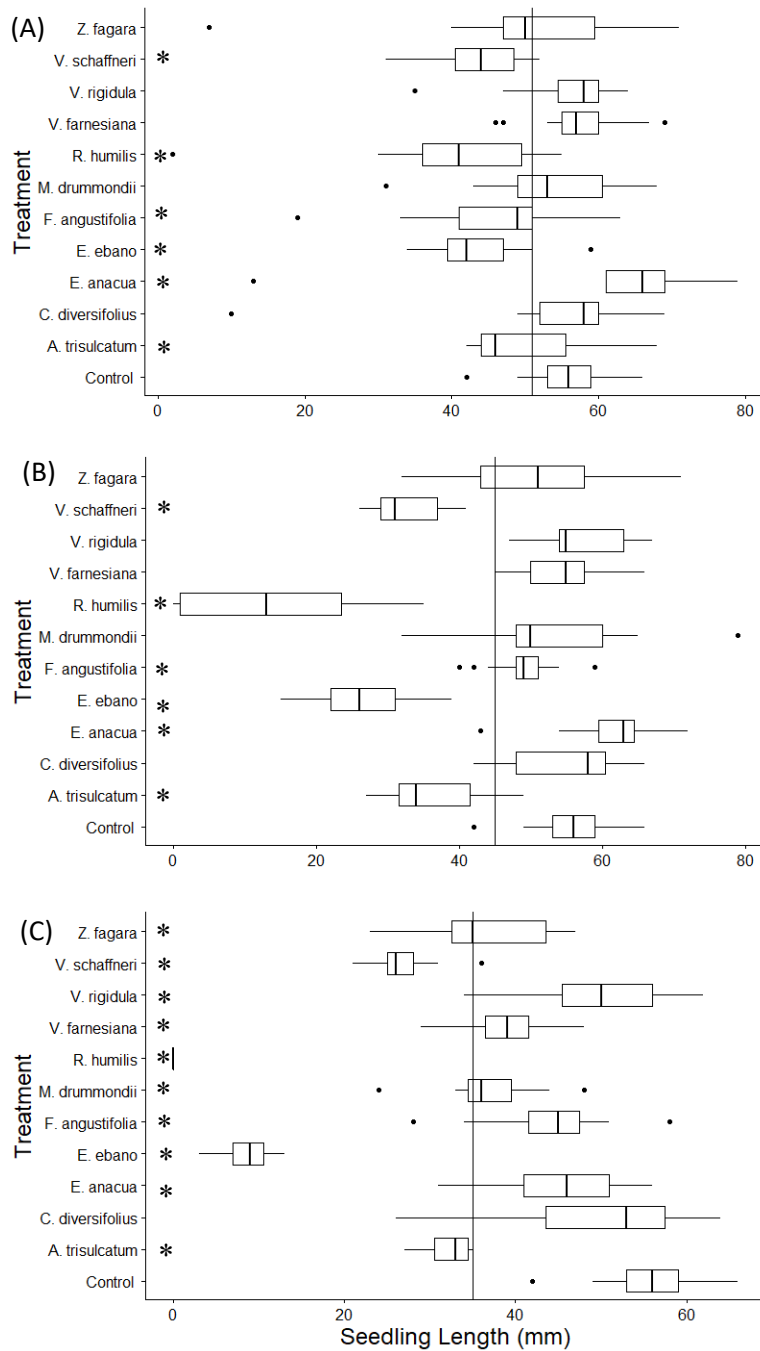


Fig. 6. Lettuce seedling length 5 days following the application of aqueous extract. The black line indicates the mean length of all seedlings at that treatment concentration (A) 33% concentration; (B) 66% concentration; (C) 100% concentration. The species with mean lengths shorter than the overall average are those that show greater allelopathic impacts than the others. * Indicates treatments that were significantly shorter than the control ($p < 0.05$).

<i>Species</i>	<i>Percent Germination</i>	<i>SGI</i>	<i>MGT</i>	<i>Length</i>
<i>Abutilon trisulcatum</i>	No	Yes	Yes	Yes
<i>Cocculus diversifolius</i>	No	No	No	No
<i>Ebanopsis ebano</i>	Yes	Yes	Yes	Yes
<i>Ehretia anacua</i>	No	No	No	No
<i>Forestiera angustifolia</i>	No	No	Yes	Yes
<i>Malvaviscus drommondii</i>	Yes	No	Yes	Yes
<i>Rivina humilis</i>	Yes	Yes	Yes	Yes
<i>Vachellia farnesiana</i>	No	Yes	Yes	Yes
<i>Vachellia rigidula</i>	No	No	No	No
<i>Vachellia schaffneri</i>	No	Yes	Yes	Yes
<i>Zanthoxylum fagara</i>	Yes	Yes	Yes	No

Table 1. Summary of the metrics in which each species exhibited allelopathic potential. Percent germination, Seed Germination Index (SGI), Mean Germination Time (MGT), and seedling elongation (Length) were the metrics used to establish allelopathy. Only the lowest concentration (33%) solution from each species was considered for this table. Yes indicates that the species exhibited allelopathy for that metric, No indicates that the species did not exhibit allelopathy.

Aqueous Extract Properties

The aqueous extracts were tested for pH and specific conductance. The pH ranged from 5.15 to 7.19 and conductance ranged from 621 to 9470 $\mu\text{S}/\text{cm}$. The pH and SPC of each aqueous solution are listed in Table 2. The pH did not correlate strongly with any of the other variables, the strongest correlation was between pH and seedling length with a correlation coefficient of -0.26, $p\text{-value} < 0.001$. SPC did correlate with three of the variables; it was negatively correlated with a correlation coefficient of -0.71, $p\text{-value} < 0.001$; and with length a correlation coefficient of -0.67, $p\text{-value} < 0.001$. SPC was positively correlated with MGT with a correlation coefficient of 0.40, $p\text{-value} < 0.001$ (Fig. 7). These correlations indicate that treatments with higher conductance resulted in reduced speed of germination, and shorter overall seedling lengths. (Fig. 6).

Species	Dilution	pH	SPC ($\mu\text{S}/\text{cm}$)
A. trisulcatum	0.33	6.4	1469
	0.66	6.4	2650
	1.00	6.45	3450
C. diversifolius	0.33	5.93	1147
	0.66	5.83	2270
	1.00	6.02	3250
E. anacua	0.33	6.54	1026
	0.66	6.61	1965
	1.00	7.14	2620
E. ebano	0.33	6.87	1254
	0.66	6.84	2300
	1.00	6.86	3160
F. angustifolia	0.33	5.15	1447
	0.66	5.15	2660
	1.00	5.41	3660
M. drommondii	0.33	6.57	2140
	0.66	6.41	3930
	1.00	6.41	5270
R. humilis	0.33	7.19	3790
	0.66	7.14	6500
	1.00	6.89	9470
V. farnesiana	0.33	6.62	922
	0.66	6.5	1652
	1.00	6.47	2150
V. rigidula	0.33	6.38	652
	0.66	6.14	1216
	1.00	6.12	1522
V. schaffneri	0.33	5.96	621
	0.66	5.62	1036
	1.00	5.55	1286
Z. fagara	0.33	6.19	1326
	0.66	6.04	2380
	1.00	6.07	3100

Table 2. The pH and Specific Conductance (SPC) of each aqueous extract.

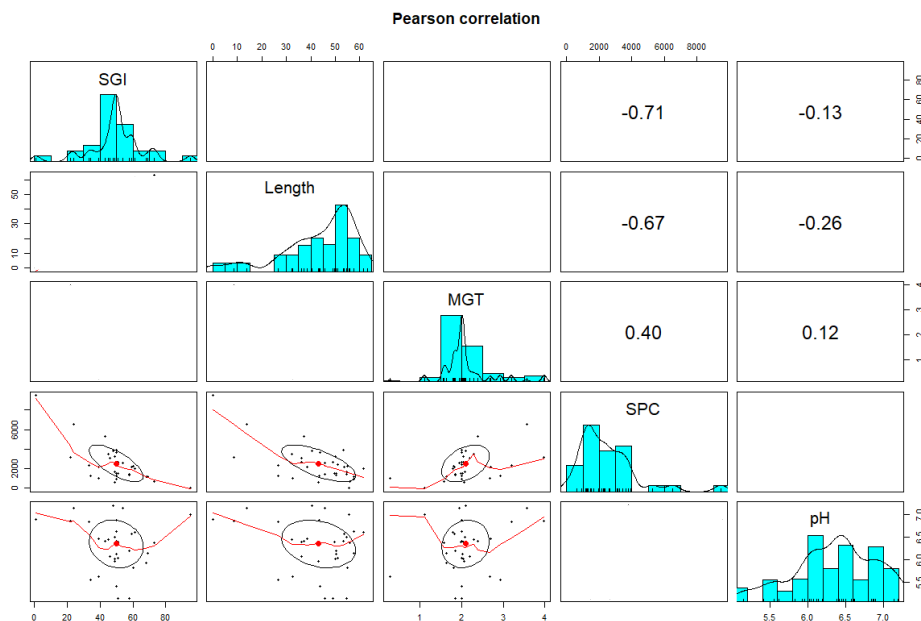


Fig. 7. Correlation matrix between the pH and Specific Conductance (SPC) of the aqueous extracts and the results of the bioassay tests. The numbers are Pearson correlation coefficients between the respective variables. The histograms represent the distribution of each variable. The scatterplots show the variable plotted against one another with a regression line and correlation ellipses. SGI and length were both highly negatively correlated with SPC, and moderately positively correlated with MGT. Length was slightly negatively impacted by pH, pH was not strongly correlated with any measurements.

Pot Experiment

Guineagrass AMF Infection

Very little AMF was found in the guineagrass roots (Fig. 8). Most samples exhibited infection though the quantities were too low to analyze statistically. There was at least one arbuscule found in each treatment with most root samples having more than one arbuscule. The roots from the control treatment did exhibit a more consistent infection, as all roots examined had AMF (Table 3). A type of fungi called dark septate endophyte (DSE) was also found in many of the roots. DSE was not detected in the *E. ebano* or *V. schaffneri* treatments.

Treatment	AMF	DSE	Comments
<i>Control</i>	present	present	AMF present on most roots in small numbers
<i>A. Trisulcatum</i>	possible	present	Not certain of AMF presence, if present it is an incredibly small amount. Less Dark Septate EMF than other treatments
<i>E. ebano</i>	present	not present	Very little AMF and not seen on all roots, only 1-2 per field of view when seen
<i>M. drummondii</i>	present	present	AMF not seen on most roots. One root had multiple AMF that were evenly distributed
<i>R. Humilis</i>	present	present	Some roots with no AMF, some areas with multiple AMF per field of view
<i>T. inula</i>	present	present	Very little AMF and not seen on all roots, only 1-3 per field of view when seen
<i>V. schaffneri</i>	present	not present	Very little AMF and not seen on all roots, only 1-2 per field of view when seen
<i>Z. fagara</i>	present	possible	AMF on all roots, not evenly distributed, some areas had higher concentrations Only one Dark Septate EMF

Table 3. Arbuscular Mycorrhizal Fungi (AMF) and Dark Septate Endophytes (DSE) presence in fine root hairs of guinea grass grown in pots treated with potentially allelopathic leaf material.

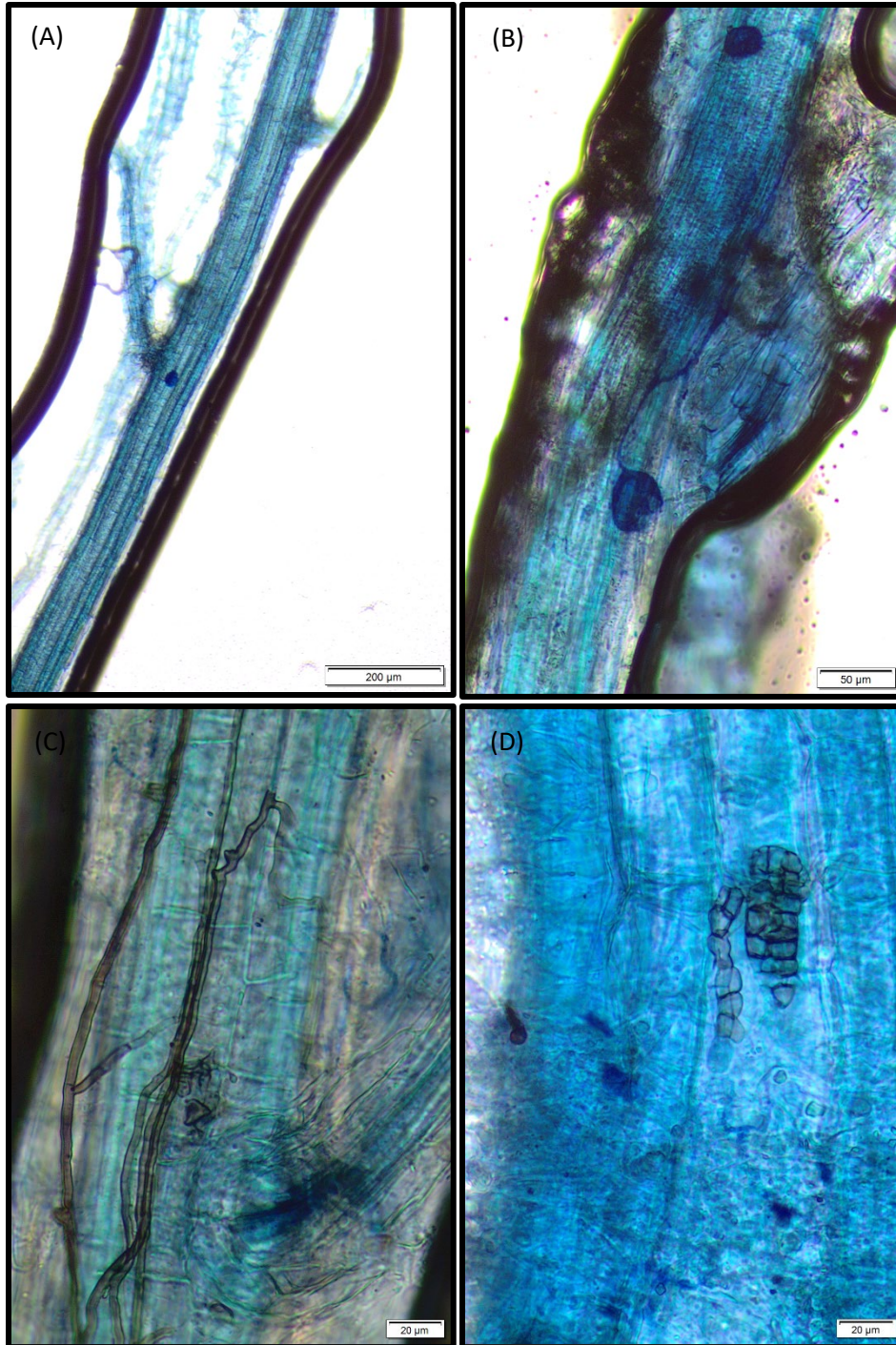


Fig. 8. Microscopic views of stained guineagrass root hairs. All treatments had low AMF colonization. The top images depict AMF infection in roots treated with leaf litter from (A) *Z. fagara* and (B) *R. humilis*. DSE was found in all treatments except *E. ebano* and *V. schaffneri*. The bottom two images show (C) DSE hyphae in guineagrass roots treated with *T. inula*, and (D) DSE microsclerotia in roots treated with *Z. fagara*.

Guineagrass Growth

A greater difference in maximum height was observed at the conclusion of the seven-week experiment than at three weeks. The maximum height ranged from 41 cm to 62 cm at week three, and at week seven the height ranged from 61 cm to 109.5 cm (Fig. 9). The height data at week three were found to be normally distributed and an ANOVA was run. The ANOVA indicated that there was no difference among the treatments p -value = 0.11 at week 3. The final height data did not fit the assumptions normality and for that reason a Kruskal-Wallis rank sum test was run, resulting in p - value = 0.17 indicating that there was no significant difference between treatments at week 7. Dry mass ranged from 4.41 g to 9.54 g with the control treatment having the lowest average dry mass at 5.88 g. (Fig. 10) This finding was contrary to what was expected because the control was the only treatment not exposed to allelopathy. The dry mass data were normally distributed, and ANOVA was run to assess variance resulting in p -value = 0.04.

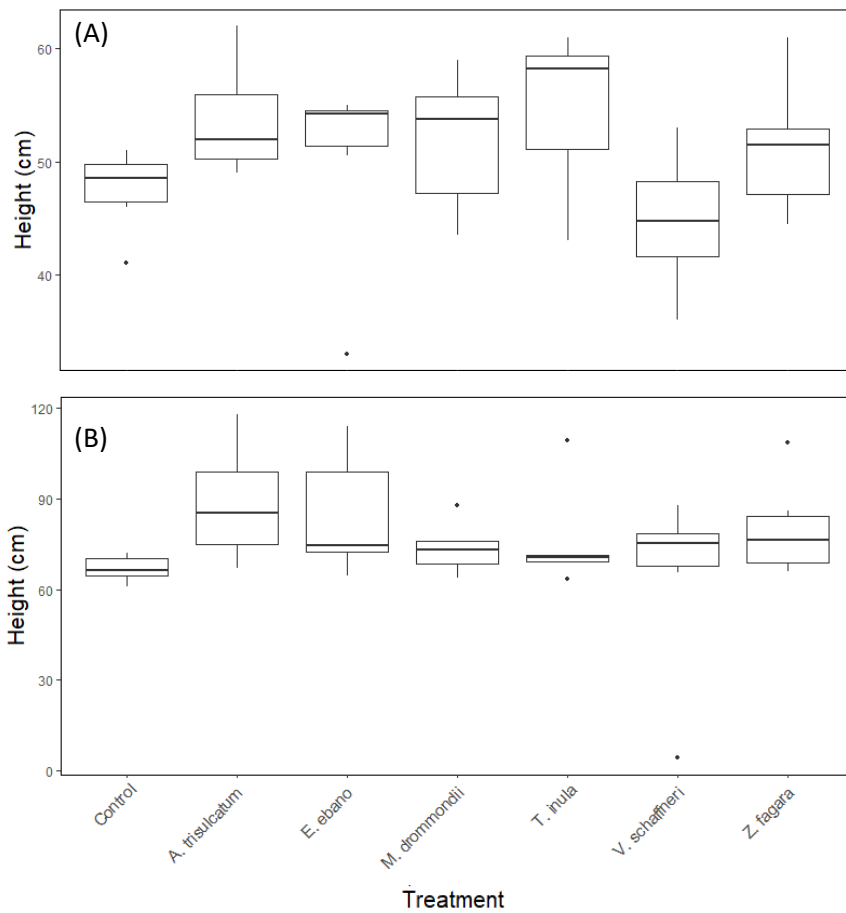


Fig. 9. Maximum height of the guineagrass in the pot experiment (A) the height 3 weeks in to the experiment; (B) the final height of the guineagrass. There was no significant difference in height between the treatments and the control. * indicates significant differences

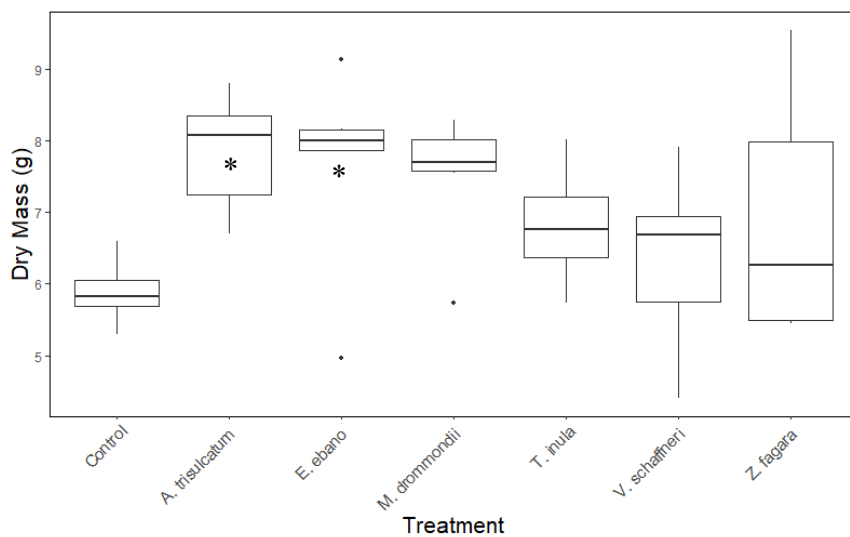


Fig. 10 Dry mass of the above ground guineagrass in the pot experiment. The control had significantly lower biomass than *A. trisulcatum*, *E. ebano*, and *M. drommondii*, and did not vary significantly from the other treatments.

Guineagrass Stem Mortality

Stem mortality refers to the number of stems in each pot that were no longer alive at the end of the experiment. The stem mortality data were normally distributed so an ANOVA was run, the p -value = 0.19 indicating that there was not significant variation between groups. When looking at the box plot of the data (Fig. 11) it did seem that there was a difference between the treatments individually and the control. For this reason, a Dunnett's test was run to compare each treatment to the control individually. The Dunnett's test did not indicate that there were significant differences.

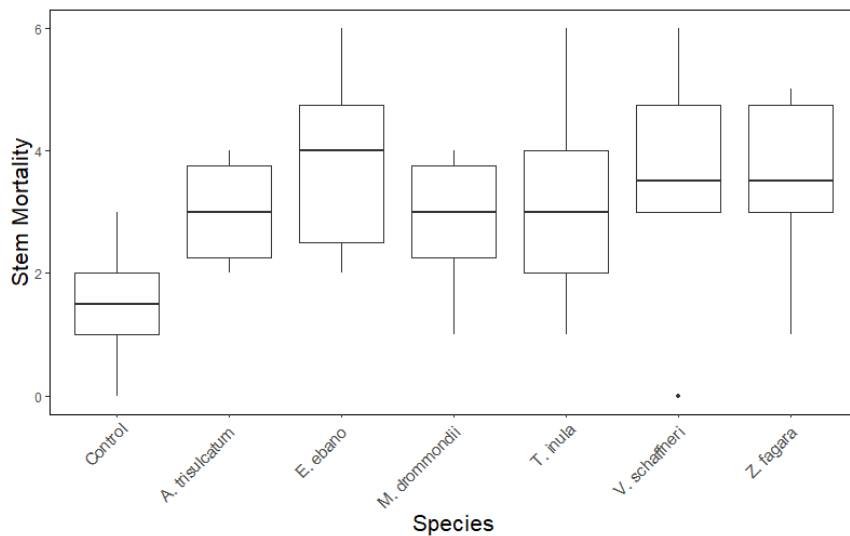


Fig. 11 Guineagrass stem mortality per pot. There were 10 stems per pot at the beginning of the experiment.

Field Experiment

Mulch Plots

Guineagrass growth was slow initially with an increase in percent cover seen in January and February that then decreased through May (Fig. 12). In August, at the conclusion of the mulch plot experiment there were statistically significant differences between the treatments. The data were not normally distributed, the data was log transformed and still did not meet the assumptions of normality, so a Kruskal-Wallis rank sum test was run, the p -value for this test

was < 0.005 . A Wilcoxon rank sum test was run on each treatment compared to the control. The *E. ebano* treatment did not vary significantly from the control, p -value = 0.12. *Z. fagara*, *E. anacua*, and *V. schaffneri* did vary significantly from the control, p -values < 0.05 .

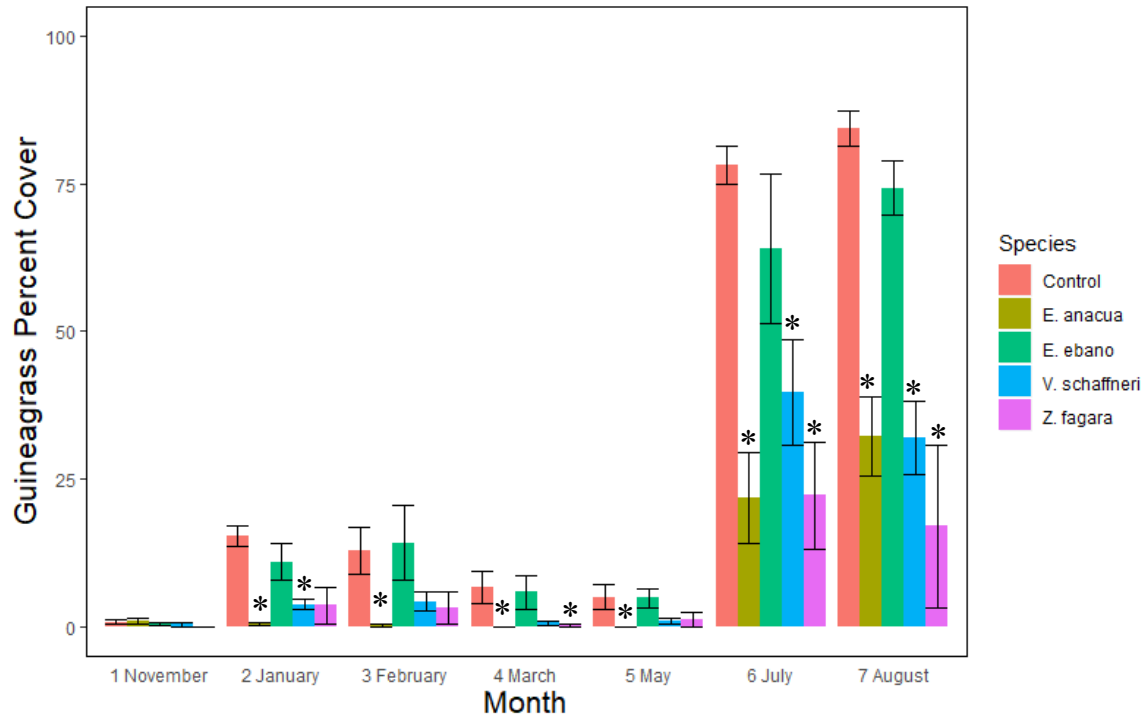


Fig. 12 Percent cover of guineagrass in the mulch plots at each sampling event. Between May and July the cover of grass increased due to increased precipitation. During the final two sampling events *E. anacua*, *V. schaffneri* and *Z. fagara* had significantly less grass cover than the control. * indicates species that differed significantly from the control. *E. ebano* treatment did not vary significantly from the control at any point during the experiment.

Standing biomass differed significantly between treatments, with the control treatment exhibiting the highest standing biomass overall, this was closely followed by *E. ebano*. *Z. fagara* had the lowest standing biomass, with three plots having no guineagrass growth at all in the inner 50 cm². See Fig. 13 for an example plot of each treatment. The standing biomass data was not normally distributed so a Kruskal-Wallis rank sum test was used to determine differences between groups, resulting in a p -value < 0.005 . A Wilcoxon rank sum test was run between the control and each treatment. *E. ebano* did not vary significantly from the control p -value = 0.55.

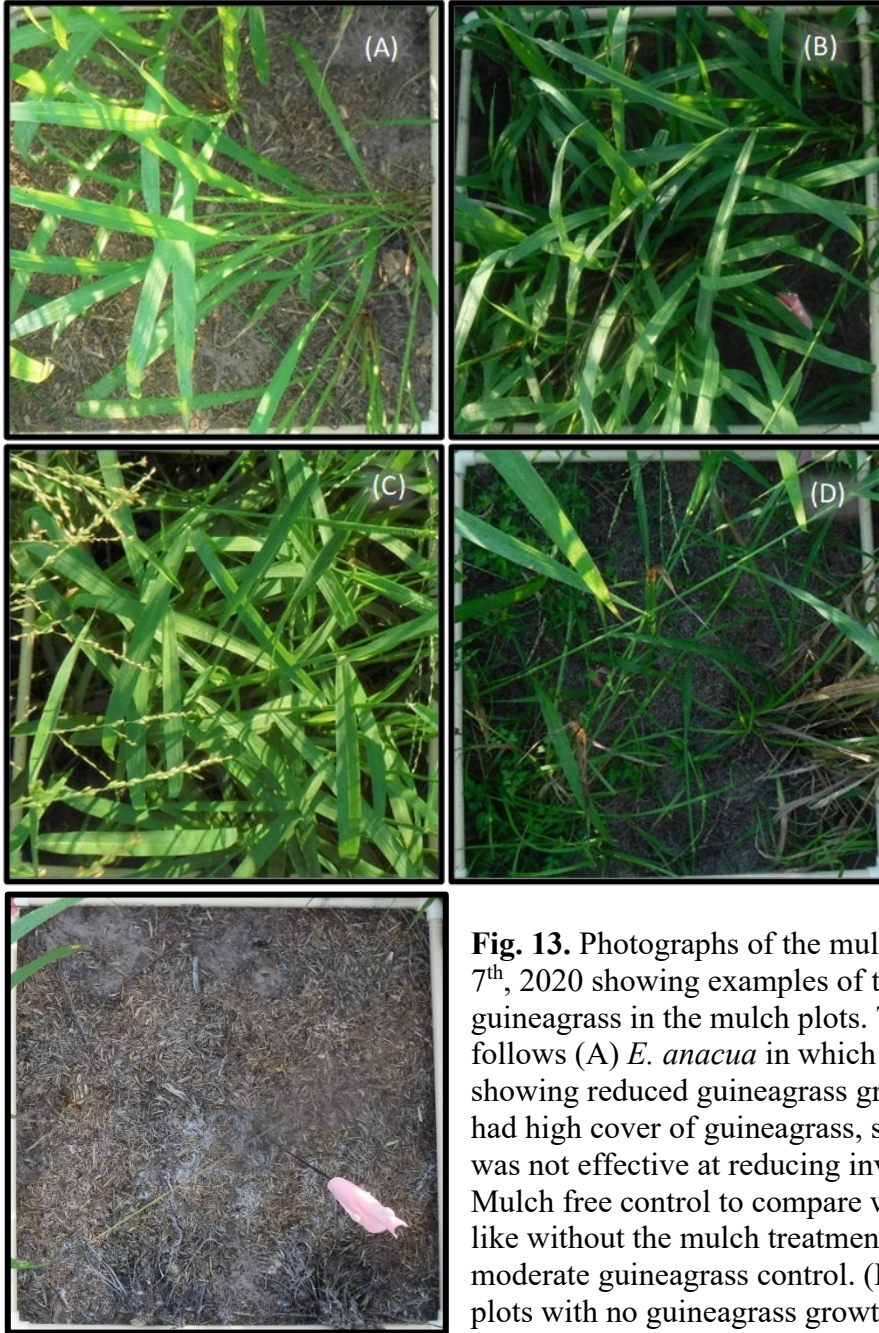


Fig. 13. Photographs of the mulch plots taken on August 7th, 2020 showing examples of the percent cover of guineagrass in the mulch plots. Treatments are shown as follows (A) *E. anacua* in which the ground is still visible, showing reduced guineagrass growth. (B) *E. ebano* which had high cover of guineagrass, showing that this treatment was not effective at reducing invasive grass cover. (C) Mulch free control to compare what the field would look like without the mulch treatments (D) *V. schaffneri* shows moderate guineagrass control. (E) *Z. fagara* which had 3 plots with no guineagrass growth in the sampling area.

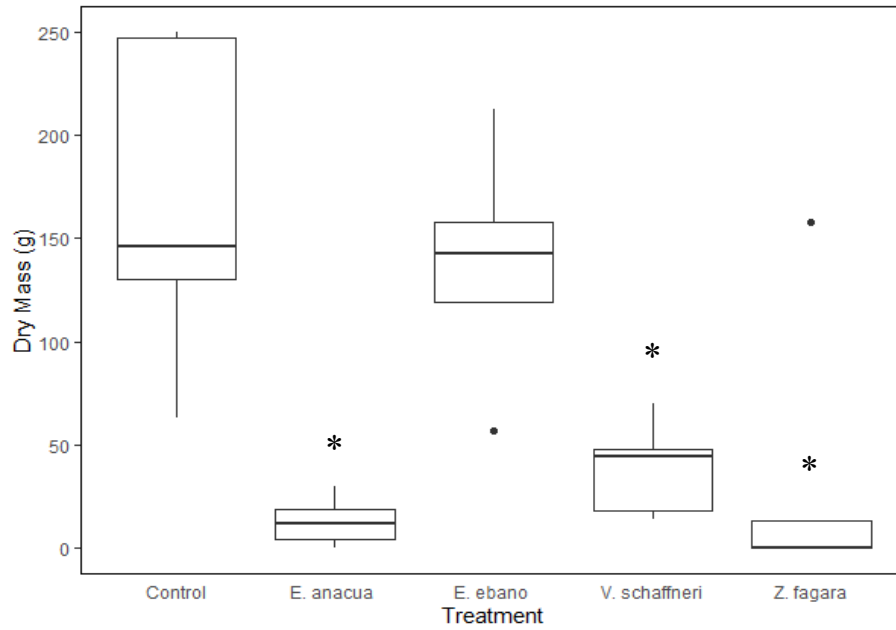


Fig. 14. Aboveground dry biomass of guineagrass in the mulch plots at the completion of the experiment, August 2020. *E. anacua*, *V. schaffneri* and *Z. fagara* had significantly less grass biomass than the control. * indicates treatments that differed significantly from the control.

Seed Plots

None of the target species germinated in the seed plots by the end of the experiment and no data was collected.

CHAPTER IV

DISCUSSION

Laboratory Bioassay

Laboratory bioassays were performed to determine if the selected native species exhibit allelopathic potential in a laboratory setting. Each treatment was compared to the water only control and to the other treatments. Species that appeared to have a greater impact on the germination process and initial seedling growth than the others were then tested in field and pot experiments. Lettuce seeds germinate rapidly and reliably; in this experiment approximately 100% germination was achieved by day six for most treatments, the exceptions being *E. ebano* and *R. humilis*. This suggests that both *E. ebano* and *R. humilis* have an impact on the germination process. Because germination of lettuce seeds is so reliable, differences between treatments were examined at germination day 3. At this point there was a visible lag in germinated seeds receiving the *E. ebano* and *R. humilis* extracts. *R. humilis* leaf extracts are known to inhibit germination of multiple species, particularly at high concentrations, with the exception of sorghum, which appears to be stimulated by high concentrations of *R. humilis* (Ravi et al., 2020). The germination process was further evaluated using SGI and MGT.

SGI and MGT are two different metrics to evaluate the speed and synchronicity of the germination process that are more sensitive than germination alone (Medina-Villar et al., 2017).

In the bioassay experiment germination of the lettuce seeds varied significantly from the control in all treatments. These observations do not necessarily imply that all treatments are allelopathic. What is shown by these tests is that some treatments have a greater impact than others, suggesting that there is a higher likelihood that they will exhibit allelopathy in the field (Shiraishi et al., 2002). Other studies have found links between allelopathic aqueous extract impacts on germination metrics. El-Kenany and El-Drier (2013) found that *Lantana canara* L. suppressed germination and reduced SGI of *Phlalaris minor* and *Sorghum bicolor*. As in this study, they saw similar increasing impacts as the concentrations of the aqueous extract was increased.

Root and shoot elongation is another aspect of early plant growth that is heavily affected by allelopathic compounds. Radicle and plumule elongation in *S. bicolor* and *P. minor* were found to be reduced when grown in petri dishes on filter paper with aqueous allelopathic extracts were added (El-Kenany and El-Drier, 2013; Wei et al., 2020). A reduction in total seedling length was seen in some treatments, those of particular note were *E. ebano*, and *R. humilis*. Other studies on lettuce seeds show impacts to radicle elongation as well, Rathinasabapathi et al. (2005) used aqueous extracts from the leaves of several woody species, the concentration of their solutions were 6.7g/L. This is approximately half the concentration of the 33% concentration (13.2g/L) solutions used in this experiment. Concentrations used in lettuce bioassay experiments vary widely between studies as there is no established concentration that mimics field conditions (Inderjit and Nilsen, 2003; Parepa et al., 2012). The 33 and 66 percent concentrations of *E. anacua* resulted in an increase in seedling length as compared to the control, indicating that some compound in the leaf extract promotes early seedling growth (cell division and elongation) following initial germination. Cummings et al. (2012) found that leaf litter from some species

facilitated the growth of the invasive grass target species. *E. anacua* was selected to be used as the control mulch treatment in the field experiment, as it was assumed that it would not negatively impact guineagrass germination and growth in the field.

The high specific conductance of the aqueous extracts, particularly *R. humilis* 100% and 66% as no other treatment had such high SPC. It would be valuable to establish at what ion concentration lettuce germination is inhibited. Initial germination and growth of seedlings is highly influenced by salinity (Katembe, 1998) and water uptake (Arteca, 1996). Imbibition in seeds and seedlings is negatively impacted by increasing concentrations of ions in the germination medium (Al-Karaki, 2007). *R. humilis* had a much higher SPC than any other treatment at its two highest concentrations, this could have contributed to the drastic reduction in germination and growth of the lettuce seeds. SPC does not appear to be an explanatory factor in the reduced growth and germination with other treatments. *M. drommondii* extracts (66 and 100% concentrations) had a SPC greater than *R. humilis* 33% concentration, neither of these treatments resulted in reduced germination speed or seedling length.

The concentration of the aqueous solution had a large impact on all testing parameters with the higher concentrations having the greatest impact, which was to be expected because higher concentration extracts contain more allelochemicals. Impacts to germination and initial growth at lower concentrations are more indicative of a species potentially expressing allelopathy in the field (Inderjit and Nilsen, 2003). The species that showed the most allelopathic potential across the bioassay experiments were: *E. ebano*, *V. schaffneri*, *V. farnesiana*, *Z. fagara*, *R. humilis*, *M. drommondii*, and *A. trisulcatum*. *V. farnesiana* was not further evaluated for allelopathy due to a lack of plant material.

Pot Experiment

Proving allelopathic interactions is notoriously challenging, as Inderjit and Calloway (2003) point out, a separation of resource competition effects from those that are chemically mediated must be achieved. Competition and allelopathic inhibition are often confounding or at least additive factors species are grown together. To rule out competition, grass plugs were grown in pots with no other plants. This may not have been enough to isolate allelopathic impacts as the height and dry mass of grass in the control treatment was lower than that of grass in the other treatments. This could be attributed to the addition of nutrients provided by the leaf litter. A lack of negative impacts during this experiment do not rule out allelopathy. It is possible that allowing the grasses to become established in the pots for 5 weeks before the addition of the leaf litter reduced the impact of the allelopathic compounds. All treatments had higher rates of stem mortality than the control, but the differences were not statistically significant.

A growing body of research suggests that one of the key ways that allelopathy works is through the alteration of the soil microbial community (Cipollini et al, 2012; Lorenzo et al., 2013; Anaya et al., 2013). Because there are so many species that rely on mycorrhizal associations, disruption of these symbioses could have a large impact on growth and survival. This experiment found no impact to the AMF infection of guineagrass roots by any treatment and, in general, the observed AMF infection of guineagrass roots was too low to analyze statistically. This could be due to the addition of fertilizer in week 4 which could have impacted colonization of fine root hairs. Liu et al. (2016) found that high amounts of phosphorus in the soil reduced root colonization by AMF in maize.

During the microscopic analysis of the roots, a type of fungi known as dark septate endophytes (DSE) was detected. All treatments but *E. ebano* and *V. schaffneri* had dark septate endophytes present. DSE seem to function as surrogates for AMF, and contribute to the overall health of the plant by increasing its access to nutrients, particularly nitrogen (Newsham, 2011). DSE is frequently found at higher altitudes and latitudes, it is possible that there is an open niche in these ecosystems as less AMF is present (Newsham, 2011) but DSE are also found in tropical environments. Santos et al. (2016) found four types of DSE in rice plants grown in the Brazilian Amazon. The presence of DSE increased the tolerance of the rice species to water stress and improved growth in plants grown with and without water deficiency. DSE and AMF do not appear to outcompete one another, research by Das and Kayang (2010) found root colonization by both types of fungi progressed synchronously and dual colonization of roots has been found in additional research (Pandey et al., 2020;) DSE colonization was found to be reduced in restored coastal dune ecosystems, in contrast AMF did not differ significantly (Gooden et al., 2020) so it is possible that DSE is more susceptible to disturbance than AMF.

Field Experiment

Growth of guineagrass in the field experiments was slow for the first seven months. This was likely the consequence of limited rainfall during this period. The first substantial amount of rain to fall during the study period occurred in June. During the month of July, hurricane Hanna hit the region, generating even more precipitation in the area and resulting in rapid growth of guineagrass in the experimental plots that were established in a previously invaded field. Between May and August, grass cover in the control treatment increased from 5% to 84%.

Unexpectedly, the *E. ebano* treatment had the highest grass cover and biomass of all the treatments with the exception of the mulch-free control. It is possible that the woody parts of this species do not contain enough of the allelopathic compounds to be effective in the field. Rathinasabpathi et al. (2005) found that extracts made from the wood of *Juglans nigra* L. (Black walnut), a tree that is known to be highly allelopathic, were not as effective at suppressing the growth of lettuce seedlings as 5 of the other species they tested showing that allelochemicals are not always found in the woody tissue of all species. The four individual plots with the lowest percent cover and standing biomass were all *Z. fagara*, the fifth *Z. fagara* plot was an extreme outlier and had been an outlier in the data since January. When that data point is excluded, *Z. fagara* had a mean grass cover of just 3% 10 months after the field was cleared and mulch applied. Stermitz et al., (1980) found 7 alkaloid compounds in *Z. fagara*, candicine, tembetarine, magnoflorine, *N*-methylisocorydine, laurifoline, chelerythrine, and trace nitidine. Alkaloids are frequently found to be allelopathic so it is possible that these compounds contribute to the effects seen (Kamal, 2011; Lebecque et al., 2018). Another factor that could have contributed to the success of this species is the presence of essential oils found in the leaves of the Rutaceae family. Sawi et al. (2019) found that essential oils found in the fruit of Rutaceae species inhibits the germination of several test species. This species shows a large amount of promise for use in guineagrass exclusion in restoration efforts in the Rio Grande Valley. *E. anacua* had been intended as the control mulch because it facilitated the growth of lettuce seeds in the bioassay experiments. In the field, *E. anacua* mulch was effective at limiting the growth of guineagrass. The final standing biomass for *E. anacua* was nearly as low as in *Z. fagara* and the final cover was 32%. *V. schaffneri* was not as effective as *E. anacua* at reducing standing biomass but resulted in the same final cover, 32%.

Mulches made from allelopathic species are frequently used to control weeds in agroforestry systems and landscaping. Wood mulches are also used to suppress weed growth; several studies have been conducted to determine if allelopathy contributes to this effect. One such study examined the impacts of aqueous extracts from *Taxodium distichum* (cypress), *Eucalyptus grandis* (eucalyptus), *Pinus celliottii* (pine), *Melaleuca quinquenervia* (melaleuca) on lettuce seeds, they also evaluated the rate of mulch decomposition and chemical properties. They found that all of the species inhibited germination of lettuce seeds, and all species except eucalyptus decayed less than 7% after one year (Duryea et al., 1999). Allelochemicals were also detected in wood chips made from *Acer rubrum* L. (red maple), *Quercus michauxii* Nutt. (swamp chestnut oak), *Juniperus siliciola* L.H. Bailey (red cedar), *Azadirachta indica* A. Juss.(neem), and *Magnolia grandiflora* (magnolia). Extracts made from wood chips of all five of these species inhibited growth and germination of lettuce seeds (Rathinasabapathi et al., 2005).

Future Studies

In nature, *E. ebano* shows very strong inhibitory effects against guineagrass. *E. ebano* in the study region is not deciduous, it does lose its leaves but there is not a large leaf drop event that occurs yearly. This species produces copious amounts of seed pods, which in natural settings can be found littering the ground beneath these trees. It would be worthwhile to perform bioassays to study the impact that these seed pods have on the growth and germination of invasive grasses, the impacts they have on the soil microbes, and the chemical compounds that they release when they decompose. Mulch from *E. anacua*, *V. schaffneri*, and *Z. fagara* should be tested against the growth of buffel grass and Kleberg bluestem. Which are the other two prominent invasive grasses in the region.

Further research should be conducted on mulch to determine how long the *Z. fagara*, *E. anacua*, and *V. schaffneri* reduce growth in order to determine the optimal mulch reapplication interval for use in thorn forest restoration. It would also be beneficial to know if the compounds break down quickly in storage conditions in order to determine how long the mulch can be stored before use without losing efficacy. Bioassays should be performed on the aqueous extracts of the mulch to determine if the mulch inhibits seed germination and growth. The impacts of the aqueous extracts should also be tested against the seeds of invasive grasses to determine which species are allelopathic against the invasive species.

It would be beneficial to attempt the seed plot experiment again. Pre-treating the seeds, irrigation of the plots, and planting during the rainy season would all likely increase the success of this experiment. Another option for this type of test would be to plant seedlings of these species to see if there is any impact to guineagrass growth when paired with higher densities of these species. Additionally, the identification of the allelopathic chemicals in each of the species should be determined to better understand these interactions.

Conclusions

Several native thorn forest species appear to be allelopathic and show potential for use in forest restoration as a means to limit invasive grass competition. Multiple species showed potential against lettuce, which is a sensitive test species. Those that showed the most potential were tested against guineagrass in pot experiments and in the field. *E. ebano*, *Z. fagara*, and *V. schaffneri* increased stem mortality in the pot experiments. In the mulch experiments *Z. fagara* mulch almost entirely suppressed the growth of guineagrass in the field for 10 months following application, and may suppress growth for longer. Mulch from *E. anacua* and *V. schaffneri*

reduced the growth of guineagrass and show potential for restoration as well. Mulch from these three species could be spread around the base of seedlings during restoration plantings to reduce competition for light, water and nutrients with invasive grasses, and at the same time, provide the usual benefits of mulch such as soil moisture conservation and soil temperature regulation.

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