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Confirmation of Allelopathic Chemicals from Reed Canarygrass (Phalaris arundinacea L.) Roots

Alexander Richard Tomes
Minnesota State University - Mankato

Confirmation of Allelopathic Chemicals from Reed Canarygrass (*Phalaris arundinacea* L.) Roots

By

Alexander R. Tomes

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This thesis has been examined and approved by the following members of the student's committee.

Dr. Bertha Proctor

Dr. Christopher Ruhland

Dr. James Rife

CONTENTS

TABLES

FIGURES

ABSTRACT

Reed Canarygrass (*Phalaris arundinacea*) is an invasive species and a major threat to grasslands and natural wetlands on nearly every landmass (Morrison and Molofsky 1998). Methanol extracts of whole and macerated Reed Canarygrass roots have been found to reduce the germination and growth of lettuce (*Lactuca sativa*), radish (*Raphanus sativus*), and the aquatic plant, Reed Mannagrass (*Glyceria maxima*) (Veit and Proctor 2009). Linoleic, linolenic and palmitic acids were identified in the methanol extracts of both the whole and macerated Reed Canarygrass roots (Proctor 2011). The purpose of this research was to determine if these chemicals individually and in combination would reduce the germination and/or growth of lettuce. Results indicate that all three compounds significantly reduce the growth of lettuce ($P<0.05$). Linolenic acid alone significantly reduced the germination. Linolenic was found to produce a statistically significant reduction in root growth at 27 ppm, followed by linoleic at 54 ppm and palmitic acid at 500 ppm. The lowest concentration tested was 27 ppm of linolenic acid. Research also reveals that when combined, linolenic and linoleic acid produce a greater reduction on growth than when treated with the compounds individually. This research proves that Reed Canarygrass has allelopathic chemicals in and on the roots.

1. Introduction

1.1. Research Justification

Proctor (2009) found that the methanol wash of whole Reed Canarygrass (*Phalaris arundinacea*; RCG) roots and macerated RCG roots reduced the germination and growth of lettuce (*Lactuca sativa*), radish (*Raphanus sativus*), and the aquatic plant, Reed Mannagrass (*Glyceria maxima*). Proctor (2011) identified three chemicals from the methanol whole root wash and methanol extracts of macerated RCG roots. These three compounds were: linolenic acid, linoleic acid, and palmitic acid.

The purpose of this research is to determine if these chemicals will reduce the germination and/or growth of lettuce. This research will also determine if the identified chemicals work better in combination. Lettuce (*Lactuca sativa*) will be used as the indicator plant. Lettuce has been shown to be very useful in trials for evaluating the varietal difference of allelopathic potential (Xuan *et al.* 2005). Germination will be recorded as mean percent germination. Growth will be measured by root length of germinated lettuce seeds.

1.2. Objectives of the Research

1. Determine if any of the three chemicals (linolenic, linoleic and palmitic acid) will individually reduce the germination and/or growth of lettuce.

2. Determine the concentration at which these chemicals reduce germination and/or growth of lettuce.

3. Determine if these chemicals work better in combination (synergistic effects).

1.3. Hypotheses to be tested

$H_{O1:}$ There is not a significant difference in percent germination of lettuce between the control seeds and the seeds exposed to chemicals identified in Reed Canarygrass (RCG) roots.

$H_{O2:}$ There is not a significant difference in lettuce root length between the control seeds and the seeds exposed to chemicals identified in RCG roots.

$H_{O3:}$ There is not a significant difference in percent germination of lettuce and root length amongst all three chemicals identified in RCG roots at the same concentrations.

$H_{A1:}$ There is a significant difference in percent germination of lettuce between the control seeds and the seeds exposed to chemicals identified in RCG roots.

H_{A2}: There is a significant difference in lettuce root length between the control seeds and the seeds exposed to chemicals identified in RCG roots.

$H_{A3:}$ There is a significant difference in percent germination of lettuce and root length amongst all three chemicals identified in RCG roots at the same concentrations.

2. Literature Review

2.1. Mechanisms of Plant Invasion

Exotic weed species, or species of weeds that are not native to a particular region, are commonly thought to be more aggressive than native weeds (Sutherland 2004). Sutherland (2004) lays out four alternative hypotheses to explain this phenomenon. Invasive species may overcome native crops due to: genetic differences, releases from native competition, releases from predation, and evolution of increased competitive ability. Because invasive species coevolved with crops in differing geographic locations, introduction to a new ecosystem may provide certain weeds with an evolutionary advantage that native crops have not had to compete with yet. It is this reason why chemicals from non-native species are hypothesized to have a superior advantage over native species.

The term allelopathy originates from the Latin words *allelon* 'of each other' and *pathos* 'to suffer' (Weir *et al.* 2004). The idea of allelopathy has been around for centuries. Theophrastus, a successor of Aristotle, was the first to write on this subject (ca 300 B.C.). He noticed the harmful effects of cabbage on a vine and suggested that these effects were caused by odors from the cabbage plants (Willis 1985). But it was not until 1937 that the term was coined by German plant physiologist Hans Molisch (Willis 1985, Kohli 1998, Albuquerque 2011). Molisch defined allelopathy as "the harmful effect of one plant upon another."

Today there are many different definitions for allelopathy, most of which include positive or negative effects of secondary metabolites on another organism (Willis 1985).

The International Allelopathy Society defines the term as "any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influence the growth and development of agricultural and biological systems." Secondary metabolites are biomolecules that are not involved in the basic metabolism of plants (Fraenkel 1959). Topics concerning allelopathy commonly refer to these secondary metabolites as 'allelochemicals.' The focus of this research will be on potential allelochemicals from RCG that exhibit harmful effects on nearby plants.

Relatively speaking, allelopathic research is a somewhat new field of study (past 30 years). Allelopathic research was initially hindered by such methodological problems as to distinguishing between allelopathy and competition for nutrients and resources (Willis 1985). There was difficulty early on in proving without doubt that a chemical produced by one plant was responsible for inhibition of another plants metabolism and growth (Weir *et al.* 2004). Studying allelopathy in natural field experiments was thought to be purely theoretical. However, with the development of more sensitive instrumentation, we now can identify and then test potential allelopathic chemicals. Development of analytical methods has led to a larger number of allelochemicals identified every year (Albuquerque *et al.* 2011). Some of the common techniques include multinuclear/multidimensional nuclear magnetic resonance (NMR), high-pressure liquid chromatography (HPLC) and gas-chromatography mass-spectrometry (GC/MS). These techniques can isolate and identify chemicals that are suspected to be allelopathic. Once chemicals are identified, they are tested on target species to confirm their allelopathic abilities (Albuquerque *et al.* 2011).

Plants that possess allelopathic abilities have a wide array of mechanisms to release allelochemicals into the environment. Different species also have varying ways of allelochemical storage. Allelochemicals have been shown to be present in leaves, bark, roots, root exudates, flowers, fruits, rhizomes, seeds and pollen (Bertin *et al.* 2003, Weir *et al.* 2004, Albuquerque *et al.* 2011). These allelochemicals can then be released into the environment through one of four mechanisms: exudation and deposition from the leaf surface through washing by rainfall, release of volatile compounds from living parts of the plant, and decay of plant residues and root exudation (Chon *et al.* 2006, Albuquerque *et al.* 2011). The chemicals being examined in this study should accumulate in the environment known as the rhizosphere by means of RCG root exudation (Proctor 2011).

The rhizosphere is the area 0 to 2 mm away from a roots surface within the soil matrix that is significantly influenced by living roots (Bertin *et al.* 2003). It has also been defined as the volume of soil influenced by root activity (Hinsinger 1998). This area is critical for growth, exudation production and development of micro and macro biota. The rhizosphere is responsible for nutrient and water uptake, exudation and rapid microbial growth (Uren 2000). It is in this area that the highest concentrations of allelochemicals should be found if the dispersal mechanisms are root exudation, as they are for RCG.

Root exudation, also called rhizodeposition (Bertin *et al.* 2003) can represent between 30 and 40% of a plant's photosynthetic productivity in the seedling stage (Whipps 1990). Root hairs, single celled extensions of the root epidermis, typically release large amounts of root exudates. Root hairs are the primary point of contact between plant and soil in the rhizosphere, and comprise as much as 77% of total root

surface area (Parker *et al.* 2000). Root exudates include numerous compounds but mainly consist of carbon-containing compounds (Uren 2000, Bertin *et al*. 2003), such as the chemicals released from RCG. The non-carbon containing compounds are comprised of H^+, inorganic ions, water and electrons.

In younger plants, the release of root exudates is very high and can make up 30% of the total dry matter production (Sauerbeck *et al.* 1981, Whipps 1990, Bertin *et al.* 2003). The rate of root exudation and the amount of exudates released primarily depends on species, cultivar, age and stress factors (Uren 2000). Root exudation rates have been shown to decrease with plant age and increase with soil stresses such as: compaction, drought, low nutrient supply, extreme temperature, and increased ultraviolet radiation (Brady and Weil 1999, Brimecombe *et al.* 2001, Uren 2000, Pramanik *et al.* 2000, Inderjit and Weston 2003, Gross 2003).

Root exudates have three primary mechanisms of entering into the rhizosphere. Depending upon weight, size and charge, root exudation may take place through diffusion, ion channel transport or vesicle transport (Bertin *et al.* 2003). Diffusion usually concerns low molecular weight organic compounds like sugars and amino acids. Ion channel transport releases exudates that cannot diffuse across the root membrane such as specific carboxylates (Bertin *et al.* 2003). High-molecular weight compounds such as flavonoids, enzymes, growth regulators, nucleotides, tannins, steroids and fatty acids are released via transport vesicles out of the root membrane (Curl and Truelove 2006, Fan *et al.* 1997, Uren 2000). The potential allelochemicals released from RCG are fatty acids, so their expected release into the rhizosphere is via transport vesicles (Bertin *et al.* 2003).

Once released, the chemicals are subjected to physical, chemical and biological processes in the soil (Chen 1995, Bertin *et al.* 2003). These three processes could alter the efficacy of the allelochemicals present before they reach their target species.

2.2. Reed Canarygrass

The geographic range of RCG in North America is presented in Figure 1. Reed Canarygrass can survive in many dynamic habitats and can be found on every land mass except Antarctica and Greenland (Hodgson 1968, Morrison and Molofsky 1998). Lavergne and Molofsky (2004) state that RCG was introduced to North America from Europe around 1850. However, Merigliano and Lesica (1998) identified collections of RCG that predate this time. The current strains of RCG in North America are thought to be a mixture of native strains and strains introduced from Europe (Merigliano and Lesica 1998). RCG is considered a "pest" species in nine U.S. states (Lavergne and Molofsky 2004), and the Minnesota Department of Natural Resources (2013) treats it as an invasive species and a major threat to natural wetlands.

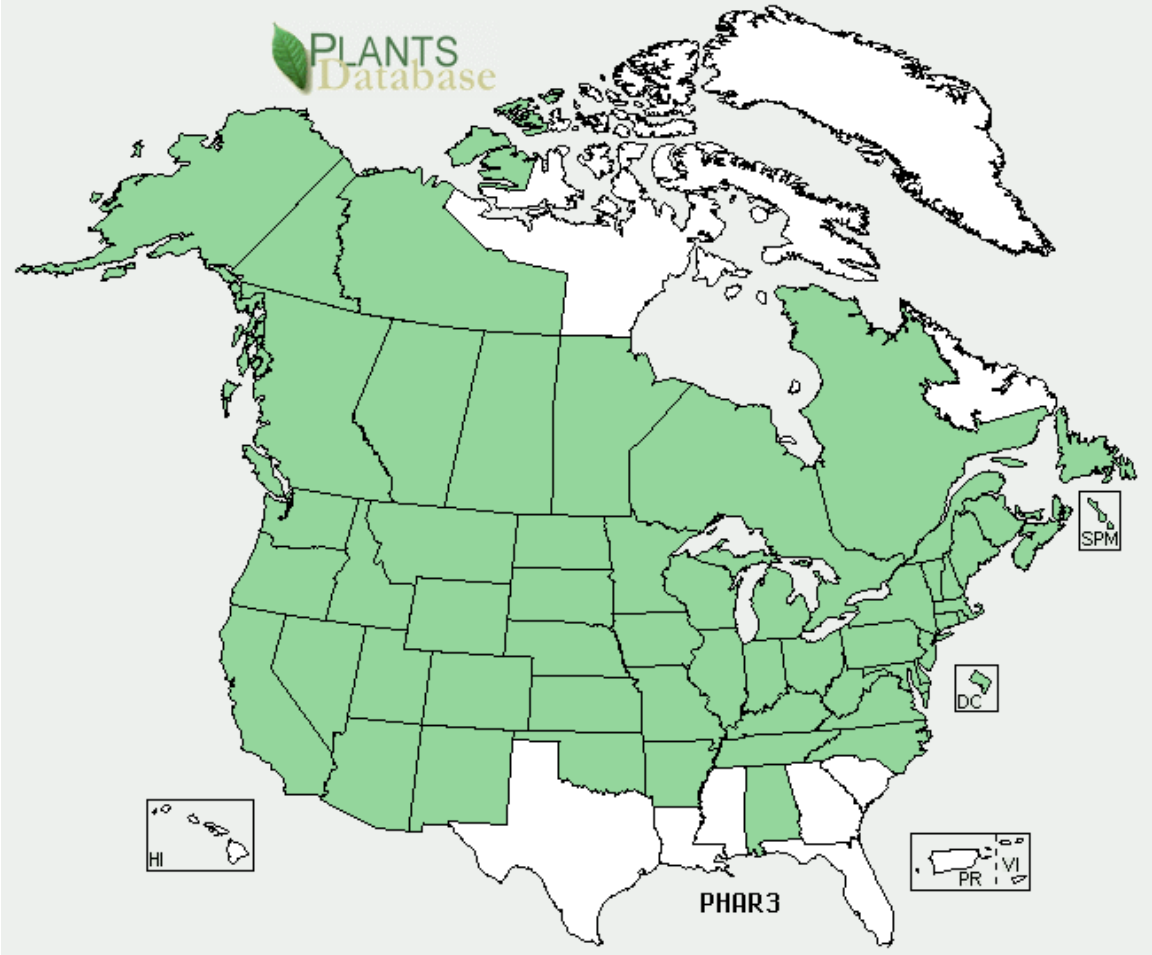

Figure 1 Geographic range of Reed Canarygrass in North America. Highlighted areas are states or providences where Reed Canarygrass presence has been confirmed. (USDA 2013)

Reed Canarygrass is a one to two meter tall, long-lived perennial grass with a C3 photosynthetic pathway (Lavergne and Molofsky 2004). It produces dense crowns, has a very high annual seed yield and has networks of vigorous underground rhizomes that allow for aggressive vegetative spread (Katterer and Andren 1999, Lavergne and Molofsky 2004). An image of the RCG seed crown is shown in Figure 2. It grows best in cool and moist conditions (Coops *et al.* 1996) and can be found in virtually any wet

habitat, including: wetlands, river banks, lake shores and floodplains (Morrison and Molofsky 1998, Lavergne and Molofsky 2004). This grass can also be found in upland sites where it can survive temporary droughts.

Figure 2 Reed Canarygrass, *Phalaris Arundinacea* L. (Invasive Plants Association of Wisconsin 2013)

Reed Canarygrass is rapidly spreading because it is frequently introduced as forage crop, perennial cover for permanent pastures, restoration of degraded soils, re-vegetation and stabilization of shorelines, wastewater treatment for removal of ammonium and nitrates and for bioenergy crop use in pulp, paper, etc. (Lavergne and Molofsky 2004). Galatowitsch *et al.* (2000) demonstrated by floristic surveys that once introduced into an environment, RCG can take over 50 – 100% of that habitat.

The ability of RCG to tolerate wide ranges in hydrology coupled with its extensive and aggressive underground network of rhizomes make this species an excellent competitor in many habitats. In addition to its physical advantages, RCG has recently been shown to demonstrate allelopathic abilities through the release of root exudates (Proctor 2011). Proctor (2011) identified three chemicals from the whole root wash and macerated roots of RCG. Those three chemicals were linolenic, linoleic and palmitic acid. Proctor (2011) also shows that there are still chemicals to be identified from RCG whole root and macerated root methanol washes. This research will determine if the chemicals identified by Proctor (2011) will reduce the germination and/or growth of lettuce.

2.3. Linolenic, Linoleic and Palmitic Acids

Linolenic, linoleic and palmitic acid are all high-molecular weight organic compounds released as root exudate fatty acids (Bertin *et al.* 2003). Linolenic (18:3) and linoleic (18:2) are both 18 carbon chain fatty acids with 3 and 2 double bonds respectively. Palmitic acid (16:0) is a 16 carbon chain with no double carbon bonds. Some of the main functions of these three fatty acids are plant growth regulation and secondary defense mechanisms (Kontos and Spyropoulos 1996, Bertin *et al.* 2003). Linolenic acid comprises >90% of the thylakoid and chloroplast membrane lipids in some plant species (McConn and Browse 1996).

Palmitic acid is one of several compounds isolated from buckwheat (*Fagopyrum spp.*) thought to be responsible for buckwheat's ability to control weed growth (Tsuzuki and Yamamoto 1987). Tsuzaki and Yamamoto (1987) identified and isolated palmitic

acid in the leaves and stems of buckwheat. Iqbal *et al.* (2003) demonstrated the ability of buckwheat to reduce growth by applying aqueous and organic solvent extracts of buckwheat's stems and leaves to lettuce seedlings. Both root and shoot growth of the lettuce seedlings was inhibited. Palmitic acid was not part of the investigation by Iqbal *et al.* (2003), but since palmitic acid has been identified in leaves and stems of buckwheat, it is suggestive that palmitic acid could be one of the responsible agents. Xuan and Tsuzuki (2004) confirmed palmitic acid as a chemical from buckwheat in a bioassay. Palmitic acid at 250 parts-per-million (ppm) has been found to significantly reduce germination of rice seedlings (Xuan and Tsuzuki 2004, Xuan *et al.* 2005).

Blooms of the algal species *Botryococcus braunii* are found in Liyu Lake, Taiwan, and are associated with phytoplankton loss and fish death (Chiang *et al.* 2004). Using 15 phytoplankton species and 5 zooplankton species, Chiang *et al.* (2004) found a close correlation between the abundance of *B. braunii* and the absence of certain phytoplankton and zooplankton. This suggests strong chemical abilities of *B. braunii* to reduce growth/development of other species. The investigators identified linolenic, linoleic, oleic, and palmitic acids as being present in the extracts of *B. braunii*. Further biological testing of each chemical identified linolenic as having the lowest median inhibitory concentration (IC_{50}) on phytoplankton growth, followed by linoleic acid and oleic acid. Palmitic acid was shown to have a much lower toxicity and thus a high IC_{50}.

Typha (cattail) is a common reed plant genus found in wetland areas throughout the United States (Gross 2003, Jarchow and Cook 2003). *Typha latifolia* (broad-leaved cattail) and *T. domingensis* (southern cattail) are both native species to the United States.

T. angustifolia (narrow-leaf cattail) is an invasive species from Europe (Stuckey and Salamon 1987, Jarchow and Cook 2003). Both *T. latifolia* and *T. domingensis* have been shown to possess allelopathic abilities (Gallardo-Williams *et al.* 2002). Aqueous extracts of *T. domingensis* have been shown to inhibit the growth of the water fern *Salvinia minima* (Gallardo *et al.* 1998). In 2002, Gallardo-Williams *et al.* isolated these extracts from *T. domingensis* and among them were linolenic and linoleic acid. Aliotta *et al.* (1990) isolated and identified linolenic and linoleic acid from *T. latifolia* as well. Jarchow and Cook (2003) found that when *T. angustifolia* was grown with the native *Bolboschoenus fluviatilis* there was a significant reduction in the leaf length, root and total biomass of *B. fluviatilis*. The combined research done on Typha shows that is it allelopathic and several species of the genus may use linolenic and linoleic acid as allelochemicals (Aliotta *et al.* 1990, Gallardo *et al.* 1998, Gallardo-Williams *et al.* 2002).

2.4. Rationale for Jasmonic Acid Incorporation

Research has demonstrated the ability of linolenic and linoleic acid to convert to jasmonic acid and its methyl ester, methyl jasmonate (MeJA, Vick and Zimmerman 1983, Vick and Zimmerman 1984, Farmer and Ryan 1992, Kontos and Spyropoulos 1996, Creelman and Mullet 1997, Gundlach and Zenk 1998, Weber 2002). Jasmonic acid and MeJA are two of the best known fatty acid-derived signals in plants today (Weber 2002). Jasmonic acid and MeJA have been shown to influence developmental processes such as growth inhibition, initiation of senescence, tendril coiling and tuber formation (Creelman and Mullet 1997, Gundlach and Zenk 1998). Both compounds have also been shown to activate a number of genes when applied exogenously. Proteins of known

function to be induced include seed storage proteins in *Brassica* and *Linum* embryos (Kontos and Spyropoulos 1996) and proteins involved in pathogen and insect resistance (Creelman and Mullet 1997). Farmer and Ryan (1990) showed that MeJA induces proteinase inhibitors that function as high molecular defense compounds. Jasmonic acid has also been shown to repress genes encoding proteins involved in photosynthesis (Creelman and Mullet 1997).

The biosynthesis of jasmonic acid in multiple plant species has been explained by Vick and Zimmerman (1983, 1984). In short, the biosynthesis of jasmonic acid occurs via the octadecanoid pathway starting with linolenic or linoleic acid in the chloroplast (Weber 2002). The 18-carbon chain fatty acids are then oxidized to 13(S)-hydroperoxide (HPOT; Gundlach and Zenk 1998). Synthesis then terminates in the chloroplast with formation of the cyclopentenone jasmonates, 12-oxo-phytodienoic acid (12-oxo-PDA) via dehydration by allene oxide synthase. The cyclopentone jasmonates then leave the chloroplast either to act as signals or to be further metabolized in the peroxisome (Weber 2002). The next step is the reduction of the cyclopentenone ring and three cycles of β-oxidation to yield jasmonic acid (Gundlach and Zenk 1998, Weber 2002). Jasmonic acid may then leave the peroxisome where it can be methylated in the cytosol to the volatile counterpart, MeJA (Weber 2002).

There are several different postulated pathways and enzymes involved in the biosynthesis of jasmonic acid from linolenic or linoleic acid (Gundlach and Zenk 1998). It is generally agreed upon that linolenic and linoleic acid can both lead to the formation of 12-oxo-PDA. 12-oxo-PDA can then lead to the formation of jasmonic acid and MeJA.

From this, we can safely conclude that linolenic and linoleic acid have the ability to form jasmonates, however the exact mechanism as to how this happens or in what tissues this may occur are still an unsettled topic.

Research has demonstrated the ability of exogenously applied precursors to jasmonic acid (i.e. linolenic acid) to result in accumulation of jasmonic acid. Farmer and Ryan (1992) applied several different precursors to the octadecanoid pathway to tomato leaves. These externally derived precursors were taken up by the plant, and resulted in accumulations of jasmonic acid. This may indicate that the availability of such precursors to the octadecanoid pathway, like linolenic acid, could determine the rate of jasmonic acid biosynthesis. This study was done using full grown tomato leaves, whether this happens in ungerminated seeds is unknown.

To test the hypothesis that linolenic and linoleic acid are converted to jasmonic acids in the endosperms of seeds, Kontos and Spyropoulos (1996) exogenously applied all three acids to ungerminated fenugreek (*Trigonella foenum-graecum*) and carob (*Ceratonia siliqua*) seeds. Once germinated, both fenugreek and carob endosperm activity of endo-β-mannanase and α-galactosidase should start increasing (Spyropoulos and Lambiris 1980). After application of linolenic, linoleic, and jasmonic acid, production of endo-β-mannanase and α-galactosidase in the endosperms of both seeds were inhibited. Jasmonic acid had the highest percent inhibition of both enzymes, followed by linolenic then linoleic acid. These results suggest that jasmonic acid does have a role in regulating post-germination growth. Furthermore, because linolenic acid was weaker than jasmonic, and linoleic weaker than that, it supports the hypothesis that

both linolenic and linoleic may be converted to jasmonic acid in the endosperms of carob and fenugreek seeds. Furthering testing of this hypothesis with other species will lead to further understanding of the roles that linolenic, linoleic and jasmonates play in post-germination processes.

2.5 Potential Uses and Importance

Weeds are a group of plants that cause many economic and social problems making it imperative that they be controlled. Weeds are highly competitive, heavy consumers of environmental resources and are invaders of farmland. Ecologically weeds are pioneers of secondary succession (Singh *et al.* 2001) although this is often overshadowed by their economic damage. Annually in the US weeds account for a 12% loss in crop yield and cost nearly $35 billion to control (Piementel *et al.* 2001). Native weeds species have co-evolved and competed with native crops for thousands of years (Cousens and Mortimer 1995), but man has created a favorable environment only to crops, so invasions are expected. Generally weeds are better colonizers, faster reproducers, and better survivors than cultivated crops (Sutherland 2004). Weeds have characteristics such as: high seed production, high vegetative reproduction, ease of dissemination of reproductive organs, long periods of seed dormancy, and seed modifications for short and long distance dispersal (Qasem and Foy 2001).

Synthetic herbicides were first used in the 1930s (Singh *et al.* 2003). These were high input and target-species oriented. DNOC (4,6-dinitro-o-cresol) was the first patented synthetic herbicide followed by the likes of 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (2-methyl-4-chlorophenoxyacetic acid). These herbicides greatly increased crop

yield by minimizing competition with weeds and competing species. However, rising costs and various environmental movements during the 1970s and 80s increased public awareness and concern over the health and safety hazards of these synthetic herbicides. Public pressure demanded that toxicological and environmental impacts be assessed on some of these herbicides and more stringent guidelines developed (Singh *et al.* 2003).

With repeated use of the same herbicides on the same sites, natural selection has favored the selection of species resistant to these chemicals (Holt and LeBaron 1990). Herbicidal resistance is becoming a growing problem throughout the world, and this brings about the need to find natural compounds that control weeds. Currently there are 272 weed-resistant biotypes belonging to 172 species (98 dicots and 64 monocots) resistant to herbicides (Holt and LeBaron 1990, Singh *et al.* 2003). Cross resistance is also developing quickly, where a weed is resistant to a chemical that is tried for the first time. These problems bring about the need for a natural herbicide from plant products. Herbicides from plant products are deemed to be safer due to their much shorter half-life (Singh *et al.* 2003). Plants that produce such natural herbicides are known as allelopathic plants. These plants release chemicals into the environment as a natural defense mechanism.

The use of allelochemicals and allelopathic plants in weed management techniques could be of great help in improving crop production and finding solutions to herbicide-resistant weeds. Allelochemicals may be used indirectly or directly as alternatives to herbicides (Macias *et al.* 1997, Kohli *et al.* 1998, Qasem and Foy 2001, Singh *et al.* 2003). So far there has been over 200 weed species that have been shown to

have allelopathic abilities (Singh *et al.* 2003). Even though these weeds commonly attack crops, their allelopathic abilities can be exploited for management of very aggressive weeds, especially in the aquatic environment. Such situations include the invasive curly pondweed (*Potamogeton crispus* L.) that can be eliminated with the introduction of needle spikerush (*Eleocharis acicularis* L.; Yeo and Fisher 1970). Allelochemicals can also be extracted and purified from such weed species, and can be used directly as a synthetic herbicide may be used.

3. Methods

3.1. Materials

Materials and chemicals used in this research are listed in Table 1. All chemicals were purchased from VWR International LLC or Sigma-Aldrich and were of the highest purity available.

Table 1 Listing of materials and chemicals used in research by company, the Chemical Abstracts Service number (CAS) and the product order number.

Product	**Company**	**CAS #**	**Product #**
7-cm filter paper	Whatman	NA	1454-070
Growth Chamber	Conviron	NA	A1000
HPLC water	Ricca	7732-18-5	9153-1
Lettuce seeds	Burpee	NA	60459A
Linoleic acid	Sigma Aldrich	60-33-3	L1376
Linolenic acid	VWR	463-40-1	200019-502
Methanol	VWR	67-56-1	BDH1135-4LG
Methyl Jasmonate	VWR	39924-52-2	101959-584
Palmitic acid	VWR	57-10-3	AAAB20322-36

3.2. Experimental Design

The experiments were carried out using 9-cm diameter glass petri dishes fitted with Whatman 7-cm filter papers. All petri dishes were washed, rinsed three times with deionized water and baked in a Thermo Scientific Thermolyne benchtop muffle furnace.

Stock solutions of each chemical were diluted in 100 ml of methanol and kept in the refrigerator between 2-4^{o}C. Specific stock solution preparation can be found under

each chemical's section in this chapter. Each treatment group was run with three or four replicates and controls. Each chemical was applied using a micropipette by dropping the required dosage amount on the filter paper in each petri dish. The dish was then left on the lab bench to allow the methanol to dry. Then 10 ml of HPLC water was added to each petri dish, and 10 lettuce seeds were added to each dish. Lids were immediately put on each dish and all dishes were put into the growth chamber. The growth chamber was set at 26 ^{o}C ± 0.5 ^{o}C with a 10 hour of light and 14 hour of dark cycle.

The dishes were checked at 24 and 48 hours. After 72 hour incubation, the dishes were removed from the growth chamber and germinated seeds were counted. The roots of germinated seeds were measured from the base of the root to the top of the root (full root and hypocotyl) to the nearest whole mm. Seeds/seedlings were not used if they became trapped under the filter paper or if dishes dried out.

3.3. Quality Control / Quality Assurance

The following QC/QA measures were followed. Prior to use, each petri dish was washed, rinsed three times with deionized water and baked at 550 ^{o}C for a minimum of one hour to remove any organics. Lettuce seeds were handled by shaking them out of the package directly into the container, or by using a pair of clean forceps. Filter paper was handled with clean metal forceps. Water and methanol used in each experiment and for dilutions was HPLC grade certified. The growth chamber used was kept at 26 ^{o}C ± 0.5 ^{o}C and equipped with an automatic alarm to notify users if temperatures exceed this range. Petri dishes used in experiments were placed randomly within the growth chamber. The time was recorded when samples were put into the growth chamber to ensure that they

were removed at 72 hours after the experiment began. Each chemical treatment group had three or four replicates and they were color coded by chemical.

The micropipette used was calibrated using 200 µl HPLC grade water and a certified analytical balance. HPLC water was pipetted into a tared dish. Water has a density of 1.0 g/ml, so 200 µl should weigh 0.200 g if the micropipette is accurate. This was confirmed prior to experimentation using the micropipette and multiple replicates.

3.4. Palmitic Acid Experiment

The palmitic acid (CAS 57-10-3; VWR: AAAB20322-36) used was a solid at room temperature in the form of white flakes. To make the 1000 ppm solution out of the 95% pure palmitic acid, 105 mg of palmitic acid was diluted in 100 ml of methanol. The treatment groups of 100, 200 and 500 ppm were diluted from this 1000 ppm stock solution. To make the 100 ppm treatment group, 1.0 ml was taken from the stock and was added to the filter paper in each petri dish, then diluted by 10 ml of water. Following the same procedure, 2 ml was taken to create the 200 ppm solution and 5 ml was taken to create the 500 ppm solution. The experimental design was followed from here.

3.5. Linoleic Acid Experiment

The linoleic acid (CAS 60-33-3; Sigma L1376) used was a liquid with a light yellow color and a density of 0.904 g/ml at room temperature (25 ^{o}C). To make the 2712 ppm solution out of the >99% linoleic acid, 300 µl of linoleic acid was diluted in 100 ml of methanol. The treatment groups of 27, 54, 108, 162, 216, and 271 ppm were diluted from this 2712 ppm stock solution. To make the 27 ppm treatment group, 100 µl was

taken from the stock solution and added to the filter paper in each petri dish, then diluted by 10 ml of HPLC grade water. Following the same procedure, 200 µl was taken to create the 54 ppm solution, 400 µl for 108 ppm, 600 µl for 162 ppm, 800 µl for 216 ppm, and 1000 µl (1.0 ml) was taken to create the 271 ppm solution. The experimental design was followed from here.

3.6. Linolenic Acid Experiment

The linolenic acid (CAS 463-40-1; VWR: 200019-502) used was a colorless liquid with a density of 0.914 g/ml at room temperature (25 ^{o}C). To make the 2742 ppm solution out of the >99% linolenic acid, 300 µl of linolenic acid was diluted in 100 ml of methanol. The treatment groups of 27, 54, 109, 162, 216 and 274 ppm were diluted from this 2742 ppm stock solution. To make the 27 ppm treatment group, 100 µl was taken from the stock solution and added to the filter paper in each petri dish, then diluted by 10 ml of HPLC grade water. Following the same procedure, 200 µl was taken to create the 54 ppm solution, 400 µl for 109 ppm, 600 µl for 162 ppm, 800 µl for 216 ppm, and 1000 µl (1.0 ml) was taken to create the 274 ppm solution. The experimental design was followed from here.

3.7. Methyl Jasmonate Experiment

The methyl jasmonate (CAS 39924-52-2; VWR: 101959-584) used was a colorless liquid with a density of 1.03 g/ml at room temperature (25 ^{o}C). To make the 1030 ppm stock solution out of the >99% methyl jasmonate, 100 µl of methyl jasmonate was diluted in 100 ml of methanol. The treatment groups of 2 and 5 ppm were diluted

from this 1030 ppm stock solution. To make the 2 ppm treatment group, 25 µl was taken from the stock solution and added to the filter paper in each petri dish, then diluted by 10 ml of HPLC grade water. Following the same procedure, 50 µl was taken to create the 5 ppm solution. The experimental design was followed from here.

3.8. Mixture One Experiment – 27 ppm Linolenic Acid plus Varying Concentration of Linoleic Acid

The first mixture of chemicals contained linolenic and linoleic acid. Both treatment groups were diluted from the previous respective stock solutions created for each acid. Each treatment group contained 27 ppm of linolenic acid. Linoleic acid concentrations varied from 27 ppm, 54 ppm, 108 ppm and 162 ppm. To make the different treatments, 100 µl of linolenic acid from the stock solution was added to each petri dish. Then, either 100 µl, 200 µl, 400 µl or 600 µl of linoleic acid was added to each petri dish to create the respective dilutions. Each petri dish was then diluted with 10 ml of HPLC grade water, and the experimental design was followed from here.

3.9. Mixture Two Experiment – 54 ppm Linolenic Acid plus Varying Concentration of Linoleic Acid

The second mixture of chemicals contained linolenic and linoleic acid. Each treatment group contained 54 ppm of linolenic acid. Linoleic acid concentrations varied from 54 ppm, 108 ppm and 162 ppm. To make the different treatments, 200 µl of linolenic acid from the stock solution was added to each petri dish. Then, either 200 µl, 400 µl or 600 µl of linoleic acid was added to each petri dish to create the respective

dilutions. Each petri dish was then diluted with 10 ml of HPLC grade water, and the experimental design was followed from here.

3.10. Statistical Analysis

All statistical tests were run on the free statistical computing software program, R (R Core Team 2013). An analysis-of-variance (ANOVA) was done to compare means for significant differences ($p<0.05$) in percent germination between controls and treated seeds and means for significant differences in root length between controls and treated seeds. A post-hoc Tukey-Honest Significant Differences (Tukey HSD) test was executed to determine which chemical is significantly different from each in mean root length and percent germination. If data did not meet the parametric criteria, a Kruskal-Wallis rank sum test was done followed by a post-hoc Pairwise-Wilcox test with a Bonferroni P-value adjustment to decrease family-pairwise error in the test.

4. Results

4.1. Palmitic Acid

All the raw germination and root length data measurements are in appendix A. Summarized in Table 2 are the mean percent seed germination and root lengths for the lettuce treated with different concentrations of palmitic acid. Summarized in Table 3 are the P-values (0.05) from the ANOVA test on the palmitic acid lettuce experiments. There were no statistically significant differences for the percent germination of lettuce between the controls and any level of palmitic acid. The only statistically significant differences occurred between the root lengths at the 500 ppm treatment compared to the others.

Table 2 Mean values and standard errors (SE) for the replicates (n=3) at different palmitic acid concentrations on lettuce seed germination and root lengths of germinated lettuce seedlings, 72 hours post-treatment.

Palmitic acid concentration	Seed germination (%)		Root length (mm)	
	Mean	SE	Mean	SE
Control	100	0.00	18.50	0.685
100 ppm	96.6	3.334	17.64	0.982
200 ppm	96.6	3.334	19.47	1.015
500 ppm	76.6	14.530	11.00	1.215

Table 3 Summary of P-values at the 0.05 confidence level from analysis of variance test (ANOVA) with post-hoc Tukey HSD for the effects of palmitic acid on percent lettuce seed germination and root length at different concentrations 72 hours after treatment. Bold face indicates significant values.

Comparison	Percent seed germination P-value	Root length (mm) P-value
0 vs. 100 ppm	0.989	0.876
0 vs. 200 ppm	0.989	0.862
0 vs. 500 ppm	0.213	< **0.000**
200 vs. 100 ppm	1.00	0.499
500 vs. 100 ppm	0.318	**0.002**
500 vs. 200 ppm	0.318	< **0.000**

4.2. Linoleic Acid

All the raw germination and root length data measurements are in appendix B. Summarized in Table 4 are the mean percent seed germination and root lengths for the lettuce treated with different concentrations of linoleic acid. Summarized in Table 5 are the P-values (0.05) from the ANOVA test on the linoleic acid lettuce experiments. There were no statistically significant differences for the percent germination between the controls and any level of linoleic acid. Root lengths were significantly different from the controls at 54 ppm. Generally, as the concentration of linoleic acid increased, there were significant differences between the previous treatment groups for root length.

Table 4 Mean values and standard errors (SE) for the replicates (n=4) at different linoleic acid concentrations on lettuce seed germination and root lengths of germinated lettuce seedlings, 72 hours post-treatment.

Linoleic acid concentration	Seed germination (%)		Root length (mm)	
	Mean	SE	Mean	SE
Control	97.5	2.500	18.57	0.412
27 ppm	95.0	5.000	17.38	0.709
54 ppm	90.0	5.774	13.07	0.650
108 ppm	90.0	5.774	11.10	0.432
162 ppm	90.0	5.774	7.72	0.434
216 ppm	95.0	5.000	6.13	0.352
271 ppm	80.0	5.774	4.04	0.266

Table 5 Summary of P-values at the 0.05 confidence level from analysis of variance test (ANOVA) with post-hoc Tukey-HSD for the effects of linoleic acid on lettuce percent seed germination and root length at different concentrations 72 hours after treatment. Bold face indicates significant values.

Comparison	Percent seed germination P-value	Root length (mm) P-value
0 vs. 27 ppm	0.999	0.533
0 vs. 54 ppm	0.943	< **0.000**
0 vs. 108 ppm	0.943	< **0.000**
0 vs. 162 ppm	0.943	< **0.000**
0 vs. 216 ppm	0.999	< **0.000**
0 vs. 271 ppm	0.255	< **0.000**
54 vs. 27 ppm	0.992	< **0.000**
108 vs. 27 ppm	0.992	< **0.000**
162 vs. 27 ppm	0.992	< **0.000**
216 vs. 27 ppm	1.000	< **0.000**
271 vs. 27 ppm	0.421	< **0.000**
108 vs. 54 ppm	1.000	0.214
162 vs. 54 ppm	1.000	< **0.000**
216 vs. 54 ppm	0.992	< **0.000**
271 vs. 54 ppm	0.816	< **0.000**
162 vs. 108 ppm	1.000	< **0.000**
216 vs. 108 ppm	0.992	< **0.000**
271 vs. 108 ppm	0.816	< **0.000**
216 vs. 162 ppm	0.992	0.540
271 vs. 162 ppm	0.816	< **0.000**
271 vs. 216 ppm	0.421	0.159

4.3. Linolenic Acid

All the raw germination and root length data measurements are in appendix C. Figure 3 shows a visual comparison between the control seeds, the 54 ppm linolenic acid treatment, and the 274 ppm treatment groups. Summarized in Table 6 are the mean percent seed germination and root lengths for the lettuce treated with different concentrations of linolenic acid. Summarized in Table 7 are the P-values (0.05) from the Kruskal-Wallis test on the linolenic acid lettuce experiments. Percent lettuce seed germination was significantly different from the controls at 54 ppm. Root lengths were significantly different from the controls at 27 ppm. As the concentration of linolenic acid increased, there were significant differences between the previous treatment groups for root length, except for 274 ppm and 216 ppm.

Table 6 Mean values and standard errors (SE) for the replicates (n=4) at different linolenic acid concentrations on lettuce seed germination and root lengths of germinated lettuce seedlings, 72 hours post-treatment.

Linolenic acid concentration	Seed germination (%)		Root length (mm)	
	Mean	SE	Mean	SE
Control	95.0	2.500	19.74	0.544
27 ppm	92.5	5.000	15.92	0.547
54 ppm	65.0	5.774	8.35	0.595
109 ppm	70.0	5.774	4.75	0.270
162 ppm	67.5	5.774	3.00	0.162
216 ppm	55.0	5.000	1.55	0.157
274 ppm	42.5	5.774	1.00	0

Table 7 Summary of P-values at the 0.05 confidence level from Kruskal-Wallis rank sum test for the effects of linolenic acid on lettuce percent seed germination and root length at different concentrations 72 hours after treatment. Bold face indicates significant values.

Comparison	Percent seed germination P-value	Root length (mm) P-value
0 vs. 27 ppm	0.999	**< 0.000**
0 vs. 54 ppm	**0.002**	**< 0.000**
0 vs. 109 ppm	**0.012**	**< 0.000**
0 vs. 162 ppm	**0.005**	**< 0.000**
0 vs. 216 ppm	**< 0.000**	**< 0.000**
0 vs. 274 ppm	**< 0.000**	**< 0.000**
54 vs. 27 ppm	**0.005**	**< 0.000**
109 vs. 27 ppm	**0.028**	**< 0.000**
162 vs. 27 ppm	**0.012**	**< 0.000**
216 vs. 27 ppm	**< 0.000**	**< 0.000**
274 vs. 27 ppm	**< 0.000**	**< 0.000**
109 vs. 54 ppm	0.984	**< 0.000**
162 vs. 54 ppm	0.999	**< 0.000**
216 vs. 54 ppm	0.707	**< 0.000**
274 vs. 54 ppm	**0.028**	**< 0.000**
162 vs. 109 ppm	0.999	**< 0.000**
216 vs. 109 ppm	0.271	**0.035**
274 vs. 109 ppm	**0.005**	**0.014**
216 vs. 162 ppm	0.471	**< 0.000**
274 vs. 162 ppm	**0.012**	**0.002**
274 vs. 216 ppm	0.471	0.237

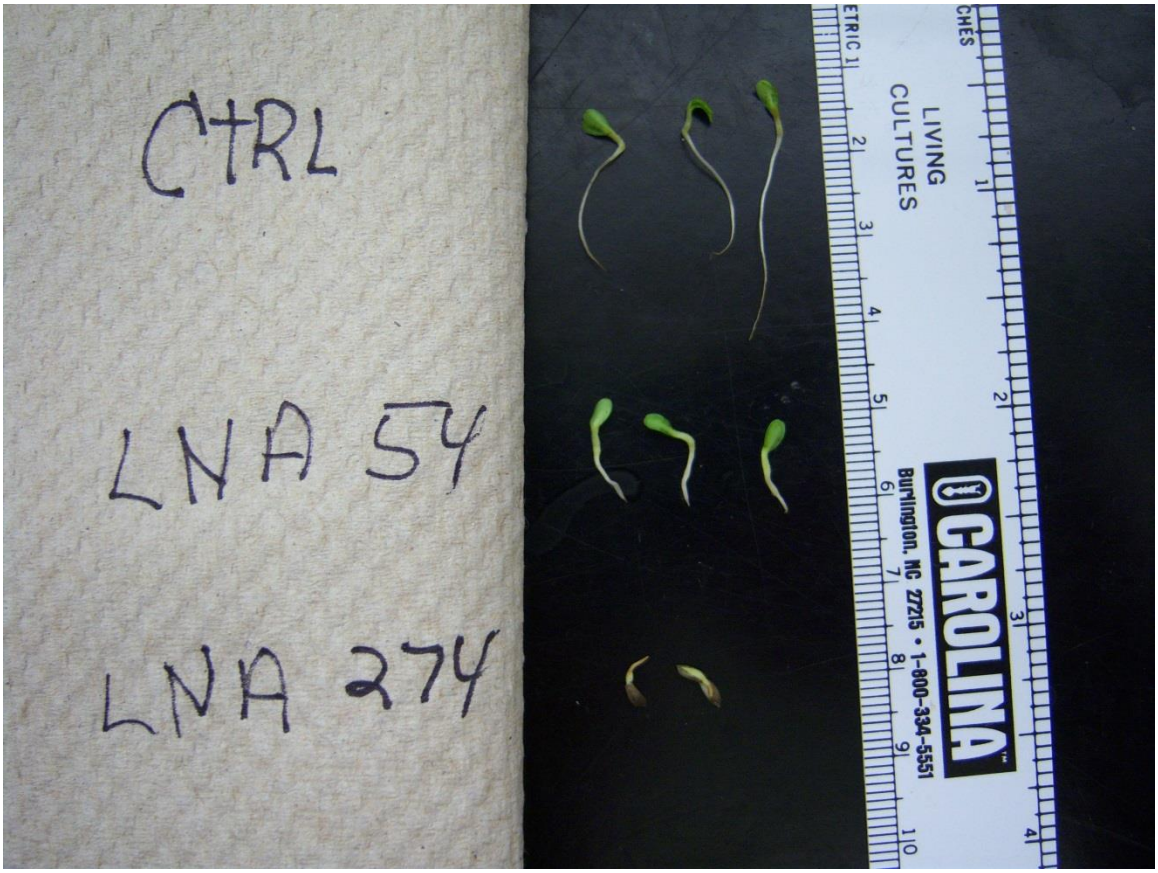

Figure 3 Germinated lettuce seedlings 72 hours post-treatment with linolenic acid (LNA) solution. From top to bottom, the treatments are control, LNA at 54 ppm and LNA at 274 ppm.

4.4. Methyl Jasmonate

All the raw germination and root length data measurements are in appendix D. Summarized in Table 8 are the mean percent seed germination and root lengths for the lettuce treated with different concentrations of methyl jasmonate. Summarized in Table 9 are the P-values (0.05) from the ANOVA test on the methyl jasmonate lettuce experiments. There were no statistically significant differences for the percent germination of lettuce between the controls and any level of methyl jasmonate. Root lengths were significantly different from the controls at 2 ppm.

Table 8 Mean values and standard errors (SE) for the replicates (n=4) at different MeJA concentrations on lettuce seed germination and root lengths of germinated lettuce seedlings, 72 hours post-treatment.

MeJA concentration	Seed germination (%)		Root length (mm)	
	Mean	SE	Mean	SE
Control	97.5	2.500	18.58	0.654
2 ppm	97.5	2.500	3.52	0.154
5 ppm	90.0	4.082	2.65	0.135

Table 9 Summary of P-values at the 0.05 confidence level from analysis of variance test (ANOVA) for the effects of MeJA on percent lettuce seed germination and root lengths at different concentrations 72 hours after treatment. Bold face indicates significant values.

Comparison	Percent seed germination P-value	Root length (mm) P-value
0 vs. 2 ppm	1.000	< **0.000**
0 vs. 5 ppm	0.257	< **0.000**
2 vs. 5 ppm	0.257	0.413

4.5. Mixture One Experiment – 27 ppm Linolenic Acid plus Varying Concentration of Linoleic Acid

All the raw germination and root length data measurements are in appendix E. The first mixture of chemicals was 27 ppm of linolenic acid added to increasing amounts of linoleic acid. Summarized in Table 10 are the mean percent seed germination and root lengths for the lettuce treated with different concentrations of the chemical mixture. Summarized in Table 11 are the P-values (0.05) from the Kruskal-Wallis test on the Mixture One lettuce experiments. There were no statistically significant differences for

the percent germination of lettuce between the controls and any level of the chemical mixture. Root lengths were significantly different from the controls at the 27 ppm mixture.

Table 10 Mean values and standard errors (SE) for the replicates (n=4) at different linoleic/linolenic mixture concentrations on percent seed germinations and root lengths of germinated lettuce seedlings, 72 hours post-treatment. Each concentration expressed was linoleic acid plus 27 ppm of linolenic acid.

Linoleic concentration plus 27ppm linolenic	Seed germination (%)		Root length (mm)	
	Mean	SE	Mean	SE
Control	97.5	2.500	18.58	0.654
27 ppm	92.5	4.787	10.72	0.535
54 ppm	90.0	5.774	8.91	0.654
108 ppm	95.0	2.887	5.96	0.435
162 ppm	95.0	2.887	4.52	0.255

Table 11 Summary of P-values at the 0.05 confidence level from Kruskal-Wallis rank sum test for the effects of the chemical mixture on lettuce percent germination and root length at different concentrations 72 hours after treatment. Concentrations expressed are linoleic acid plus 27 ppm of linolenic acid. Bold face indicates significant values.

Comparison	Percent seed germination P-value	Root length (mm) P-value
0 vs. 27 ppm	0.896	< **0.000**
0 vs. 54 ppm	0.676	< **0.000**
0 vs. 108 ppm	0.991	< **0.000**
0 vs. 162 ppm	0.991	< **0.000**
54 vs. 27 ppm	0.991	0.296
108 vs. 27 ppm	0.991	< **0.000**
162 vs. 27 ppm	0.991	< **0.000**
108 vs. 54 ppm	0.896	**0.002**
162 vs. 54 ppm	0.896	< **0.000**
162 vs. 108 ppm	1.000	0.398

4.6. Mixture Two Experiment – 54 ppm Linolenic Acid plus Varying Concentration of Linoleic Acid

All the raw germination and root length data measurements are in appendix F. The second mixture of chemicals was 54 ppm of linolenic acid added to increasing amounts of linoleic acid. Summarized in Table 12 are the mean percent seed germination and root lengths for the lettuce treated with different concentrations of the chemical mixture. Summarized in Table 13 are the P-values (0.05) from the Kruskal-Wallis test on

the Mixture Two lettuce experiments. Percent lettuce seed germination was significantly different from the controls at the 108 ppm mixture. The concentration higher than 108 ppm was not significantly different from the controls for germination. Root lengths were significantly different from the controls at the 54 ppm mixture.

Table 12 Mean values and standard error (SE) for the replicates (n=3) at different linoleic/linolenic mixture concentrations on percent seed germinations and root lengths of germinated lettuce seedlings, 72 hours post-treatment. Each concentration expressed was linoleic acid plus 54 ppm of linolenic acid.

Linoleic concentration plus 54ppm linolenic	Seed germination (%)		Root length (mm)	
	Mean	SE	Mean	SE
Control	97.5	2.500	18.58	0.654
54 ppm	90.0	5.774	4.63	0.278
108 ppm	73.3	3.334	3.10	0.315
162 ppm	93.3	3.334	2.94	0.262

Table 13 Summary of P-values at the 0.05 confidence level from Kruskal-Wallis rank sum test for the effects of the chemical mixture on lettuce seed percent germination and root length at different concentrations 72 hours after treatment. Concentrations expressed are linoleic acid plus 54 ppm of linolenic acid. Bold face indicates significant values.

Comparison	Percent seed germination P-value	Root length (mm) P-value
0 vs. 54 ppm	0.498	< **0.000**
0 vs. 108 ppm	**0.005**	< **0.000**
0 vs. 162 ppm	0.848	< **0.000**
108 vs. 54 ppm	**0.057**	**0.004**
162 vs. 54 ppm	0.927	**0.002**
162 vs. 108 ppm	**0.023**	0.997

5. Discussion

The International Allelopathy Society (1996) defines allelopathy as "any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influences the growth and development of agricultural and biological systems." A plant is allelopathic if it has the ability to influence the germination and/or growth of nearby organisms. If the chemicals released by those plants are found to inhibit the germination and/or growth of another organism, we then define those chemicals as allelochemicals.

5.1. Palmitic Acid

Palmitic acid did not significantly reduce percent lettuce germination at any concentration up to 500ppm (Figure 4). At the concentration of 500ppm the percent germination decreased to a mean of 80%, but was not significantly different ($P<0.05$) from the controls. Xuan and Tsuzuki (2004) reported that at 250 ppm palmitic acid significantly reduced the germination of rice seedlings.

Root length was significantly reduced by palmitic acid at 500 ppm (Figure 5). The mean root length of lettuce seedlings were decreased by 7.5 mm and were 41% shorter than the controls. This validates that palmitic acid has an impact on lettuce growth. Germination was not significantly reduced, but small reductions did take place at the highest concentration tested.

Palmitic acid is a known chemical found in the leaves and stems of the buckwheat plant (Tsuzuki and Yamamoto 1987, Xuan and Tsuzuki 2004). Buckwheat has a strong ability to suppress the emergence of nearby weeds and in bioassays palmitic acid was confirmed to be one of the chemicals present in the plant (Iqbal *et al.* 2003). Because of

this, it was hypothesized that palmitic acid may be one of active chemicals responsible for the weed suppression by buckwheat. The research here indicates that palmitic acid does not significantly reduce the germination of lettuce seedlings up to a 500ppm solution.

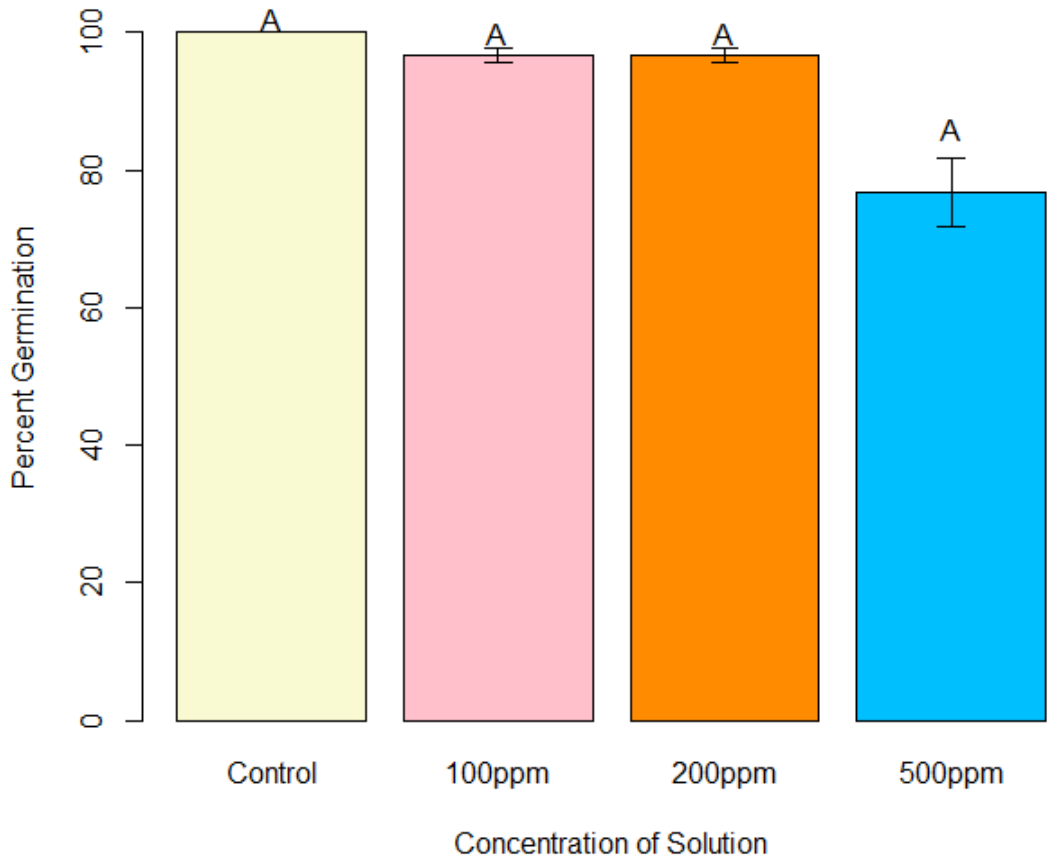

Figure 4 Percent germination of lettuce seeds 72 hours after treatment with palmitic acid chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

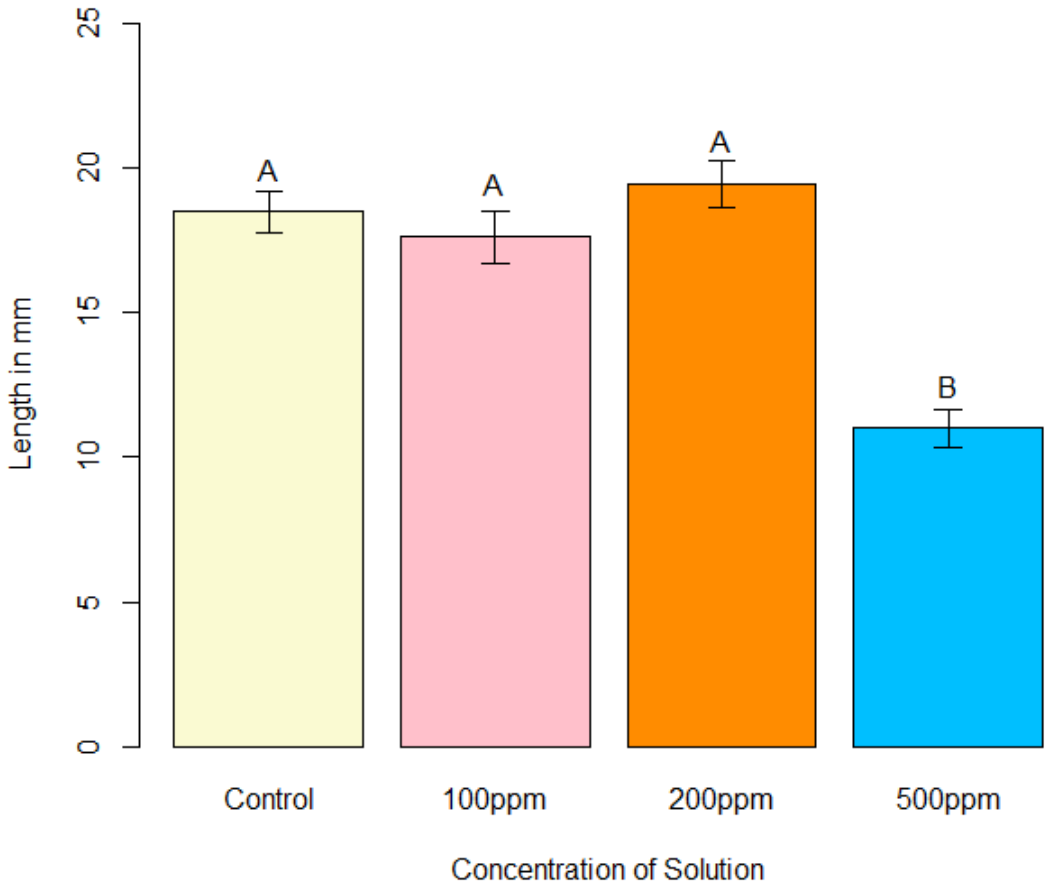

Figure 5 Germinated lettuce seedling root length 72 hours after treatment with palmitic acid chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

5.2. Linoleic and Linolenic Acid

Because linolenic and linoleic acid are frequently found together in plants (Aliotta *et al.* 1990, Gallardo-Williams *et al.* 2002, Chiang *et al.* 2004) they will be discussed together. As summarized in Figure 6, there were no statistically significant ($p<0.05$) differences between the controls and any treatment level of linoleic acid on lettuce germination. Only 80% of the seeds germinated at the 271ppm treatment level, but there was a wide variation.

As summarized in Figure 7, linoleic acid was found to significantly reduce lettuce root growth at 54 ppm. The lower concentration tested, 27 ppm, did slightly reduce the mean root length, but not by a statistically significant amount ($p<0.05$). 108 ppm of linoleic acid was not significantly different from the 54 ppm group, but generally higher concentrations showed greater reductions in lettuce root growth. This experiment confirms that linoleic acid has a significant effect on growth of lettuce seedlings.

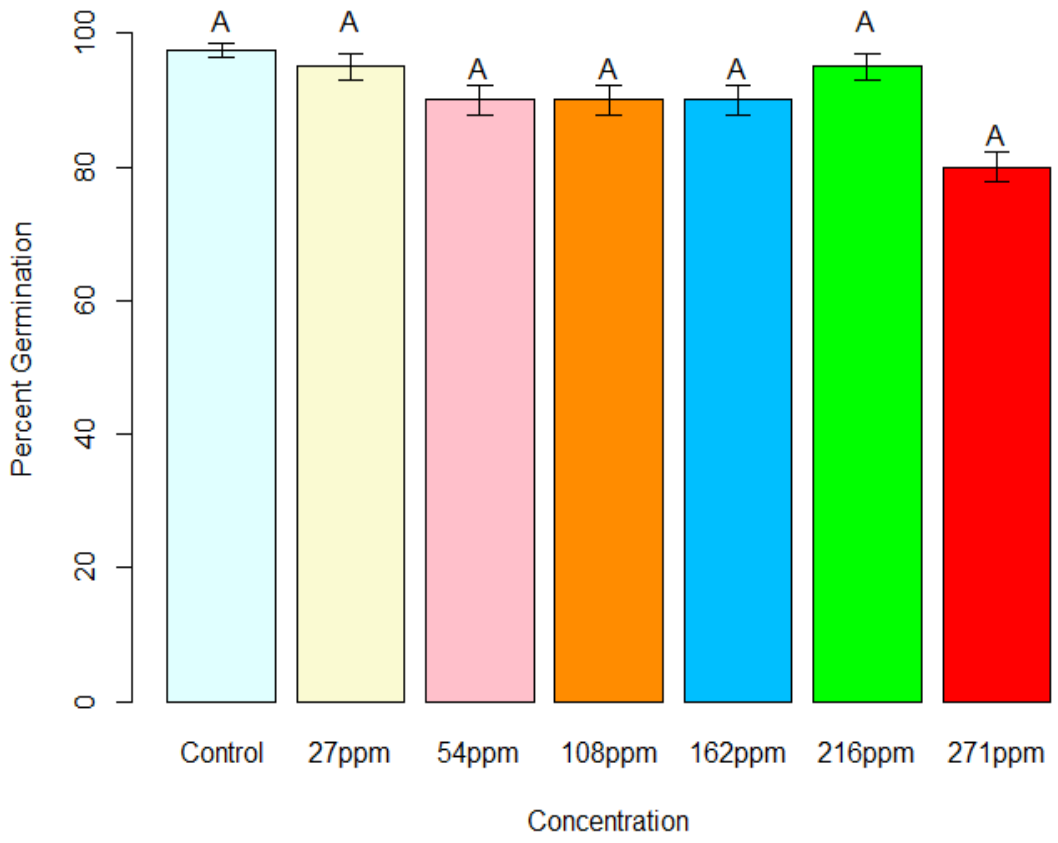

Figure 6 Percent germination of lettuce seeds 72 hours after treatment with linoleic acid solution. Differences in letters indicate a significant difference at the 0.05 confidence level. Error bars represent ±1 standard error.

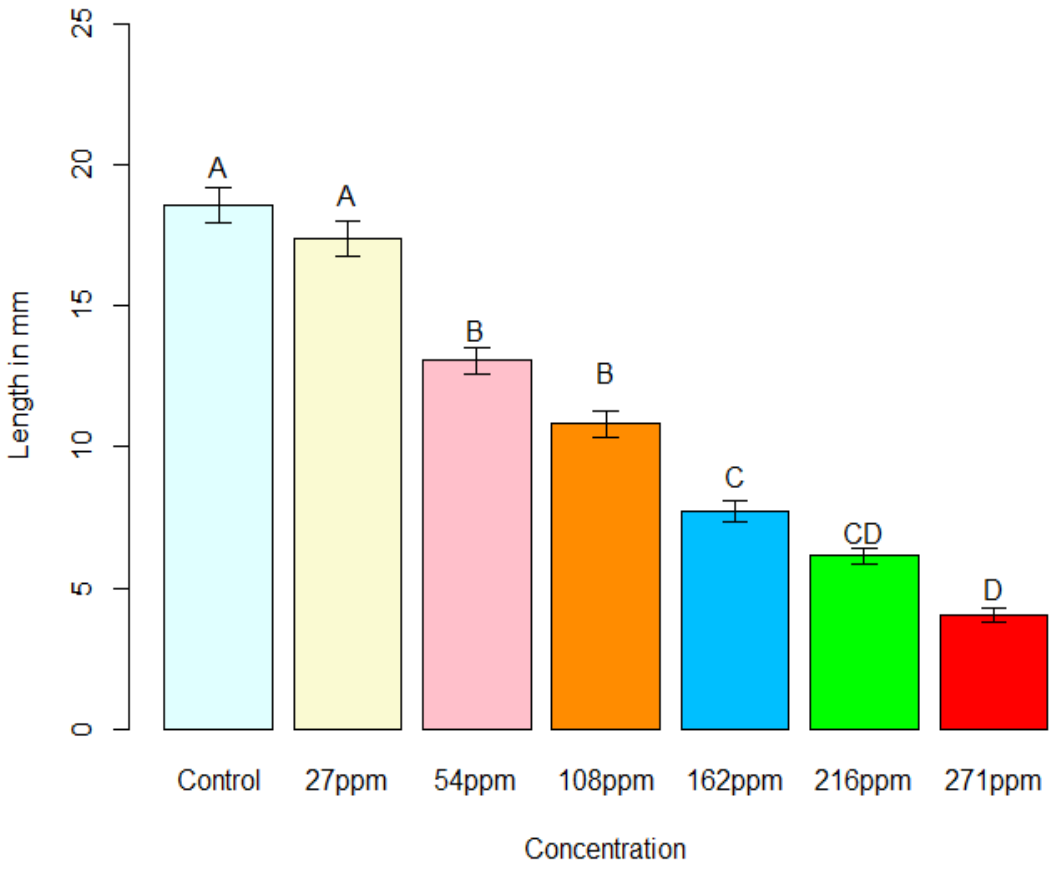

Figure 7 Germinated lettuce seedling root length 72 hours after treatment with linoleic acid solution. Differences in letters indicate a significant difference with 0.05 confidence. Error bars represent ±1 standard error.

Summarized in Figure 8 are the percent germinations of lettuce after treatment with various levels of linolenic acid. Linolenic acid was the only chemical tested that significantly reduced lettuce percent germination. Interestingly, percent germination was reduced at 54 ppm linolenic acid and not significantly reduced at 27 ppm. This suggests that not only can RCG reduce root length growth, but also germination. Since linolenic acid was the only chemical from RCG to reduce germination, we can safely assume that this is the active chemical responsible for RCG's ability to reduce germination. There are however, still more chemicals to identify from RCG roots (Veit and Proctor 2009).

As shown in Figure 9, linolenic acid was found to significantly reduce lettuce root growth at 27 ppm. Linolenic acid impacted lettuce at the lowest level tested and thus had the largest impact on lettuce root growth as compared to the other two chemicals from RCG roots. At 54 ppm, the effect is nearly doubled, to a mean length of only 8.35 mm (Table 6). Compared to linoleic acid (Figure 10), linolenic acid had a more profound effect on lettuce growth. This experiment showed that lettuce germination is inhibited at 54 ppm of linolenic acid and root growth is inhibited at 27 ppm.

Because linolenic acid had a much more profound effect on growth, this supports the hypothesis that linoleic acid may act through linolenic acid after desaturation (Kontos and Spyropoulos 1996, Gundlach and Zenk 1998). If linoleic acid is desaturated by removing two hydrogens to form a double bond, it is then linolenic acid. Not all, if any, of the linoleic acid will become desaturated. If then linoleic acid does act through the conversion to linolenic acid, it would be expected to have a less profound effect of growth as compared to direct application of linolenic acid.

Linolenic and linoleic acids both function as plant growth regulators and take action in secondary defense mechanisms (Bertin *et al.* 2003). Linolenic and linoleic acid have also been shown to makeup >90% of the thylakoid and chloroplast membrane lipids in some plant species (McConn and Browse 1996). Chiang *et al.* (2004) identified linolenic and linoleic acids as two of the primary chemicals in the algal species *Botryococcus braunii. B. braunni* is associated with high phytoplankton loss and fish death. Testing by Chiang *et al.* found linolenic acid to have the largest impact on phytoplankton loss, followed by linoleic acid. This was also confirmed here, as linolenic acid was consistently more powerful than linoleic. The allelopathic *Typha* genus has also shown to contain linolenic and linoleic acid in the root extracts. Gallardo-Williams *et al.* (2002) isolated and identified the two chemicals from *T. domingensis* and Aliotta *et al.* (1990) did the same with *T. latifolia*. Toxicity or concentrations of the two chemicals in these species have not been elucidated.

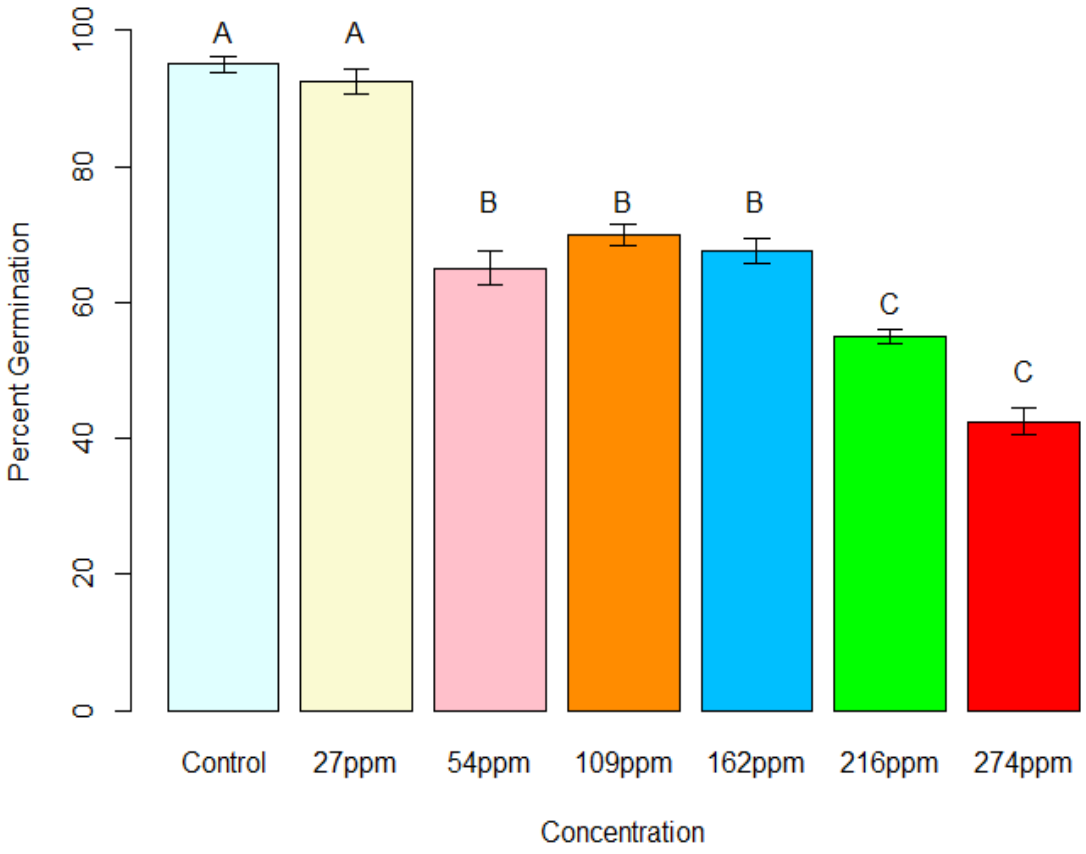

Figure 8 Percent germination of lettuce seeds 72 hours after treatment with linolenic acid solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

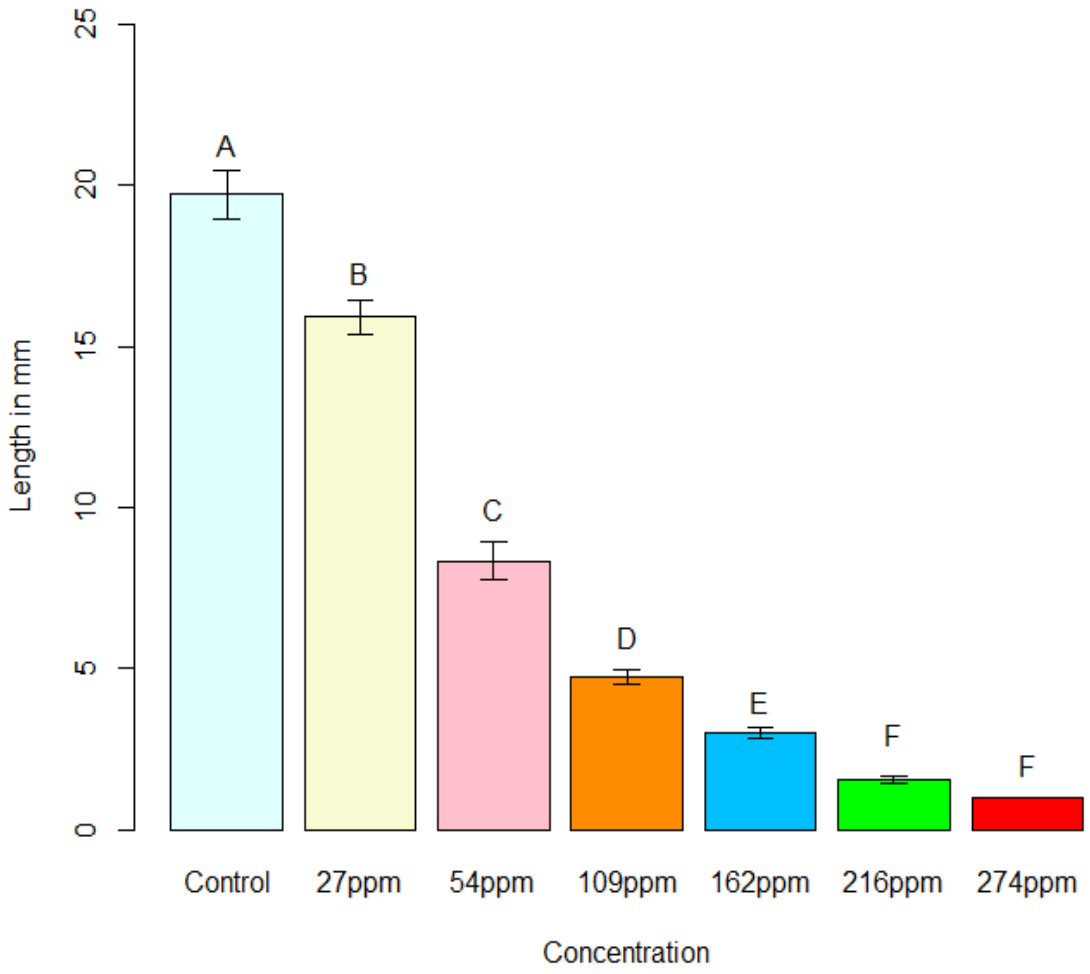

Figure 9 Germinated lettuce seedling root length 72 hours after treatment with linolenic acid solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

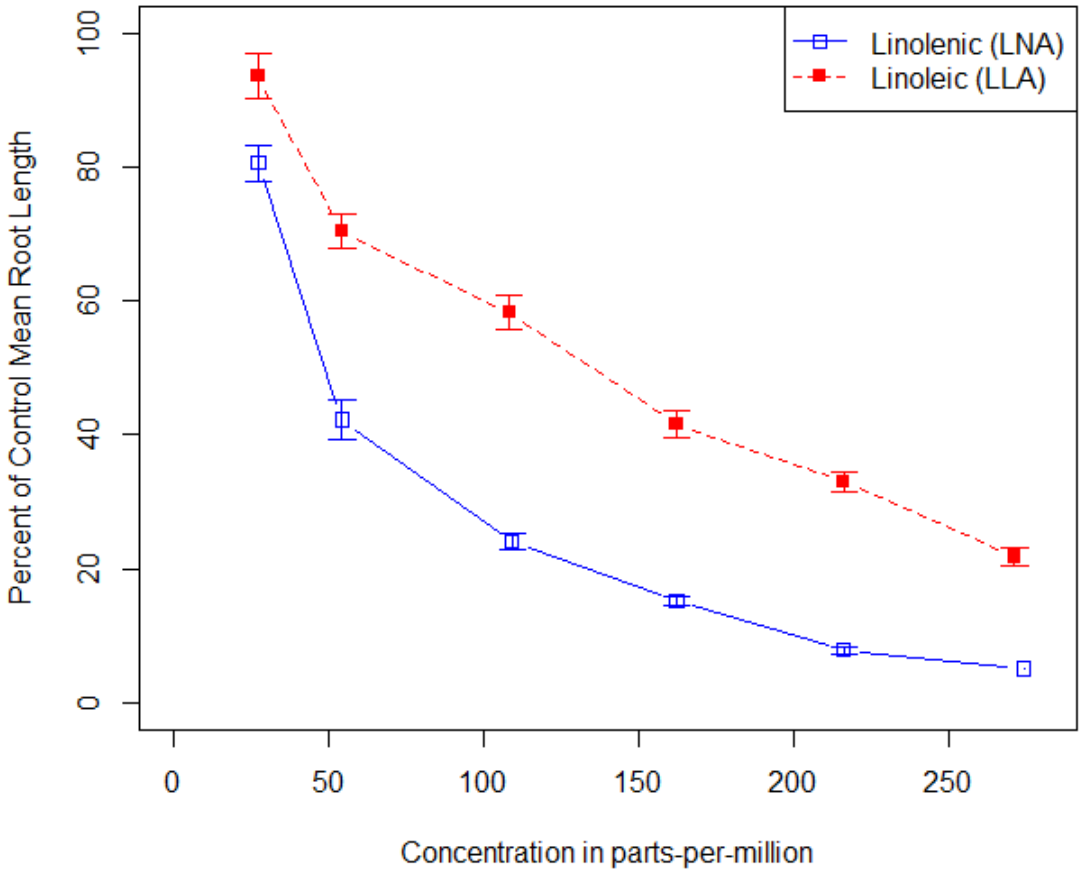

Figure 10 Germinated lettuce seedling root length expressed as a percentage of the control for seeds germinated in different concentrations of linolenic acid (LNA) and linoleic acid (LLA). Error bars represent ±1 standard error.

5.3. Methyl Jasmonate

Methyl jasmonate (MeJA) had no significant reductions in germination (Figure 11), but did show strong reductions in root growth. This supports the conclusion that the physiological effects MeJA has on lettuce are confined to post-germination.Lettuce root length was significantly reduced by MeJA at 2ppm (Figure 12). MeJA proved to have the largest impact on lettuce root growth. The 2 ppm solution of MeJA resulted in a seedling that was less than 20% of the controls. The 5 ppm solution of MeJA was not significantly different than the 2 ppm solution, with a difference of only ≈1 mm.

Methyl jasmonate is one of the most studied fatty acid derived signals in plants (Weber 2002), and it may be possible that RCG indirectly uses the jasmonates as defense compounds. Because linolenic and linoleic acid can be converted to the jasmonate family, it was decided to include the jasmonates in this bioassay. MeJA was the compound of choice, as it is the last stop in the octadecanoid pathway. This is assuming that jasmonic acid leaves the peroxisome to become methylated before leaving the cell. Literature on the topic has demonstrated the ability of linolenic and linoleic acid to convert to jasmonic acid and its methyl ester, MeJA (Vick and Zimmerman 1983, Vick and Zimmerman 1984, Kontos and Spyropoulos 1996, Creelman and Mullet 1997, Gundlach and Zenk 1998, Weber 2002).

A working hypothesis on the subject of chemical toxicity is that linoleic and linolenic acid work through the jasmonates after conversion through the octadecanoid pathway (Kontos and Spyropoulos 1996). Because the effect of linolenic acid is less than that of MeJA, it may imply that linolenic acts through MeJA. And, since linoleic acid's

effect is less than that of linolenic, it may work through MeJA as well, after desaturation to linolenic acid (Kontos and Spyropoulos 1996, Gundlach and Zenk 1998). The question still remains as to whether linoleic and linolenic acids can be converted to MeJA within ungerminated seeds. Weber (2002) outlined the conversion of linolenic acid to the jasmonates in the octadecanoid pathway, which starts in the chloroplast. Since seeds usually have no chloroplasts, this may imply that linolenic and linoleic are not converted to MeJA until after germination, if at all.

More research needs to be done on the pre and post-germination activities of these three chemicals. It may be possible to build support for this hypothesis, if we could inhibit an enzyme within the octadecanoid pathway. With the octadecanoid pathway interrupted, we could then run similar experiments to see if the reductions in lettuce growth caused by linolenic and linoleic acid still take place.

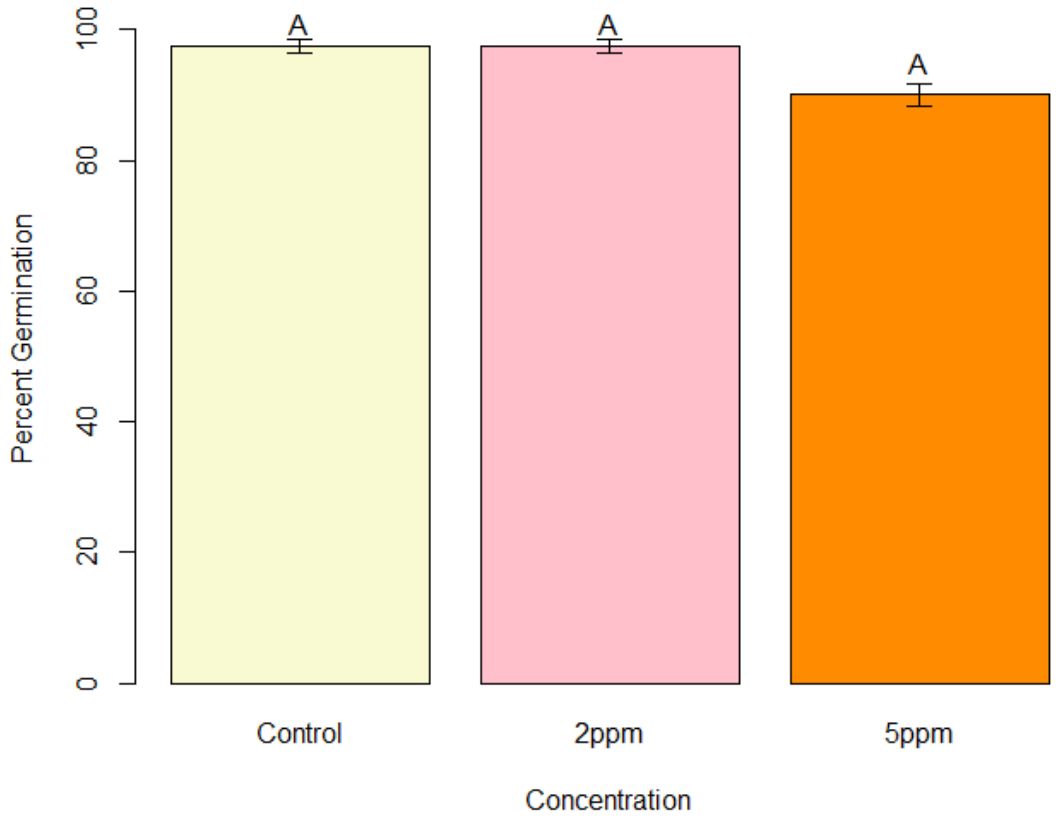

Figure 11 Percent germination of lettuce seeds 72 hours after treatment with methyl jasmonate chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

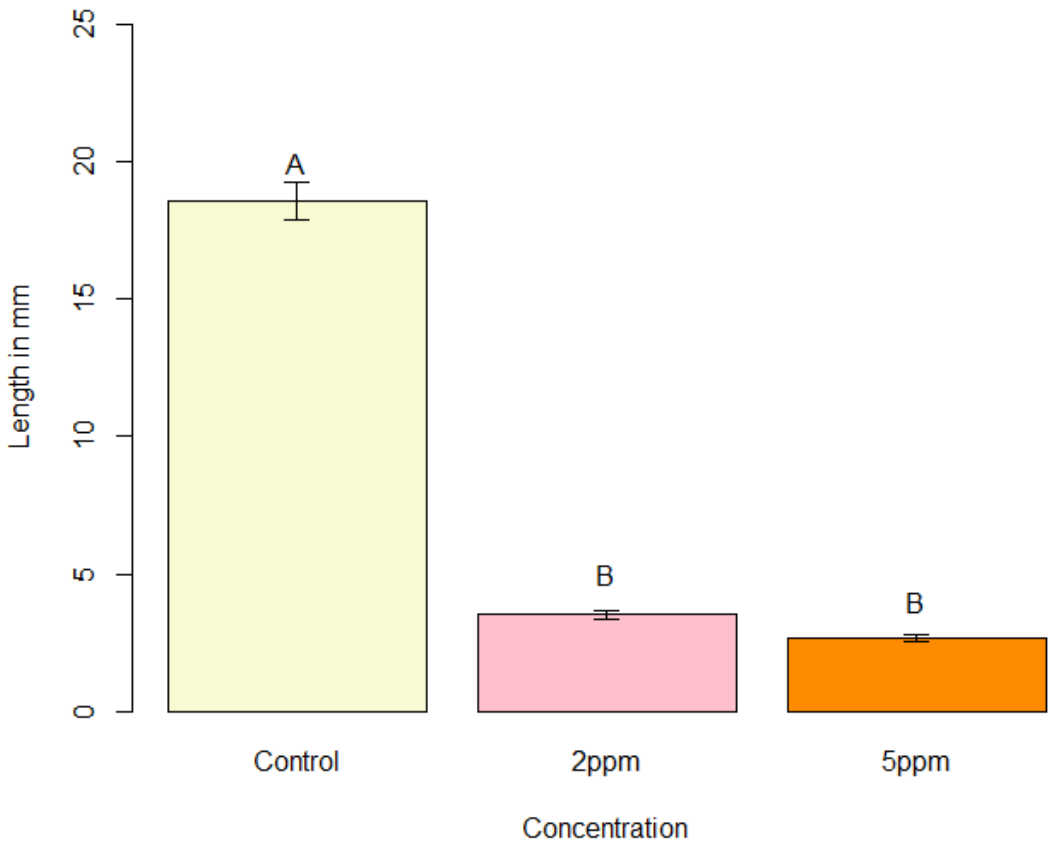

Figure 12 Germinated lettuce seedling root length 72 hours after treatment with methyl jasmonate chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

5.4. Mixture One Experiment

Mixture One experiment contained 27 ppm of linolenic acid added to varying concentrations of linoleic acid. 27 ppm of linolenic acid was the lowest-observed-effect concentration (LOEC) on growth, and 27 ppm of linoleic acid was a no-observed-effect concentration (NOEC) on growth.

As shown in Figure 13, there were no statistically significant differences in lettuce germination percent between the controls and any level of Mixture One. 27 ppm of linolenic acid tested individually had no significant effect on germination (Figure 8), and linoleic acid tested at any concentration also had no significant effect on germination (Figure 6). Because the two chemicals had no significant reductions when tested individually or in combination, it appears that the chemicals do not have any synergistic effects, as it pertains to germination.

Mixture One experiment showed significant reductions in lettuce root growth at every combination (Figure 14). The reductions in the first treatment group of 27 ppm linolenic acid with 27 ppm linoleic acid represented a stronger effect on root growth then with each chemical tested individually (Figure 17). This trend did not remain though, as increasing concentrations of linoleic acid did not prove to be more powerful than linolenic tested individually. However, each combination in the Mixture One experiment did have a stronger effect than with linoleic treatment alone. Because linoleic concentrations continually increased, and linolenic concentrations stayed the same, this supports the hypothesis that these chemicals may be synergistic.

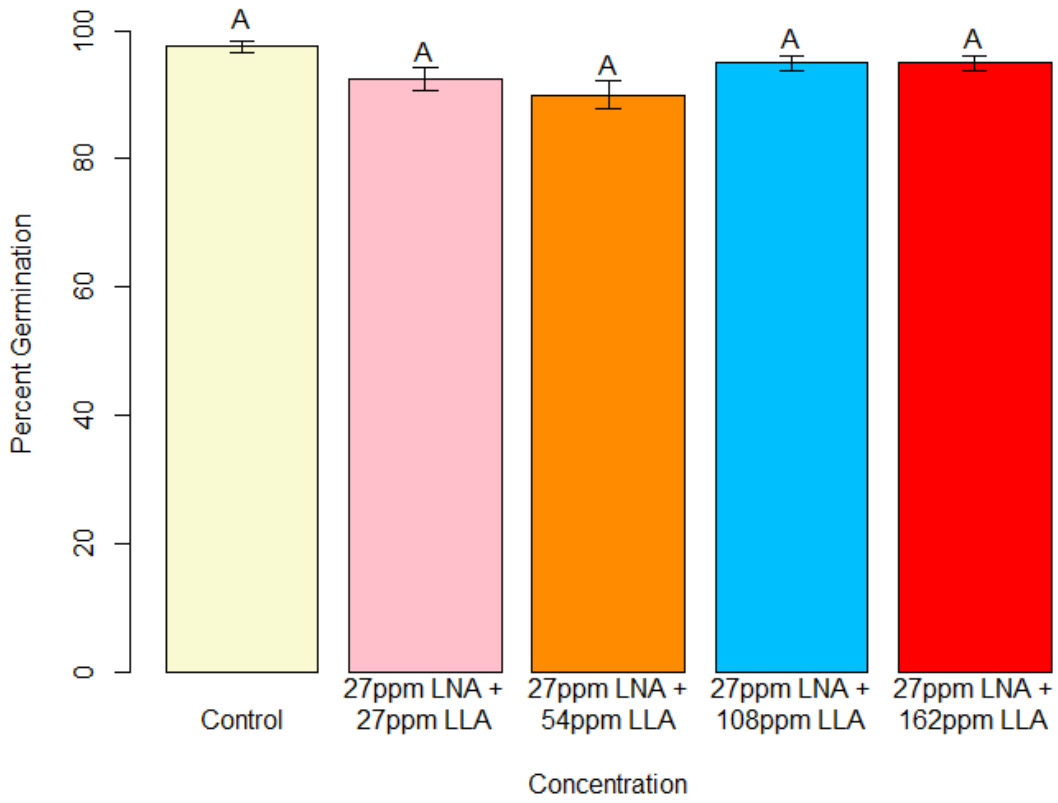

Figure 13 Percent germination of lettuce seeds 72 hours after treatment with linolenic (LNA) and linoleic (LLA) acid Mixture One chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

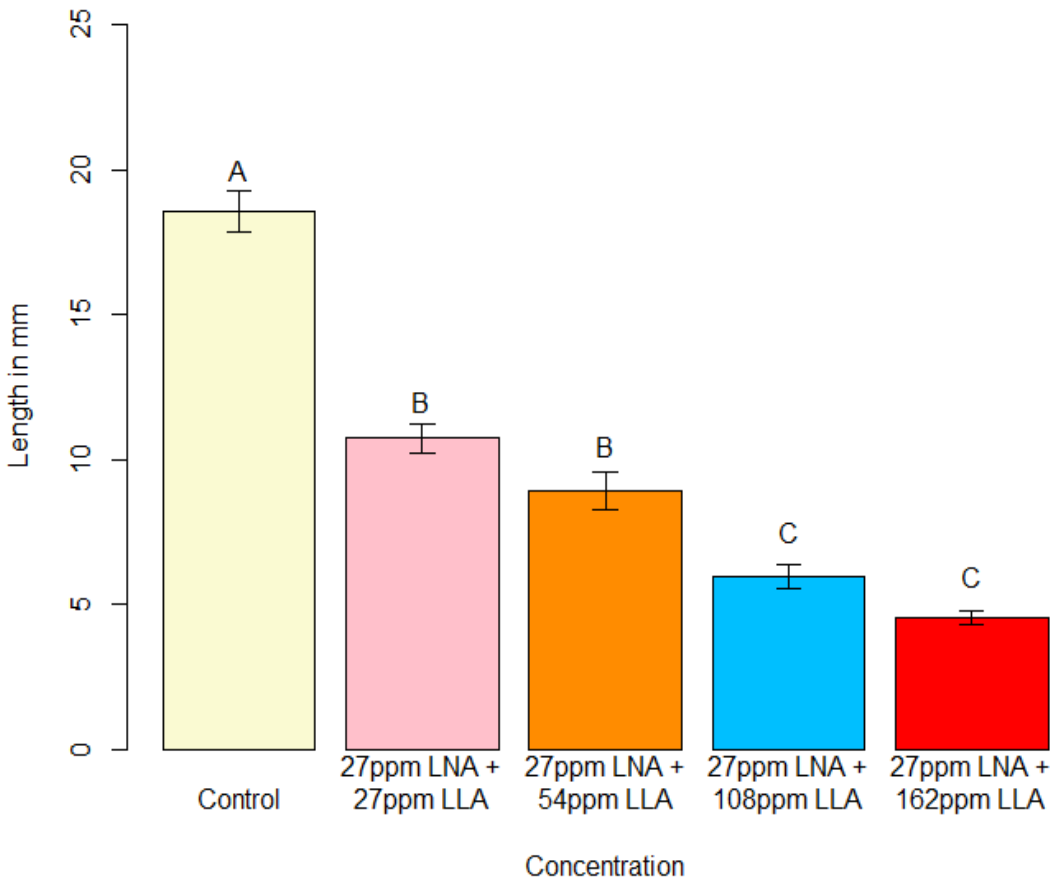

Figure 14 Germinated lettuce seedling root length 72 hours after treatment with linolenic (LNA) and linoleic (LLA) acid Mixture One chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

5.5. Mixture Two Experiment

Mixture Two experiment contained 54 ppm of linolenic acid added to varying concentrations of linoleic acid. 54 ppm of linolenic acid tested individually had significant reductions on percent germination and root growth. 54 ppm of linoleic acid represents the LOEC on root growth and no concentrations of linoleic acid had significant reductions on percent lettuce germination.

Mixture Two experiment significantly reduced percent lettuce germination, but only at 54 ppm of linolenic acid and 108 ppm of linoleic acid (Figure 15). This was unusual because the treatments lower and higher than this were not significantly different from the controls. Linolenic acid significantly reduced lettuce germination rates at 54 ppm and higher concentrations when tested individually (Table 7, Figure 8). Because linolenic acid tested individually significantly reduced lettuce germination, these results may suggest that linoleic acid has a positive effect on germination rates. Root length decreases have been consistently synergistic for mixtures; this does not stay true for germination. Linoleic acid does not affect germination by itself, and it seems to lessen the effects of linolenic on germination. More testing on combinations of these two acids may need to take place in order to fully understand the effects that they have on germination.

Root lengths at all concentrations in the Mixture Two experiment were significantly different from the controls (Figure 16). The first concentration in the experiment of 54 ppm linolenic acid and 54 ppm linoleic acid had a mean root length less than 5 mm. This represents a length less than 30% of the control group (Figure 17). The first concentration also was significantly different from both linolenic and linoleic acid

tested individually at 54 ppm. The second concentration of 54 ppm linolenic acid and 108 ppm of linoleic acid was also significantly lower than linolenic acid tested individually at 108 ppm (Figure 17). These results indicate again that there may be synergistic effects with linolenic and linoleic acid application on root growth reductions.

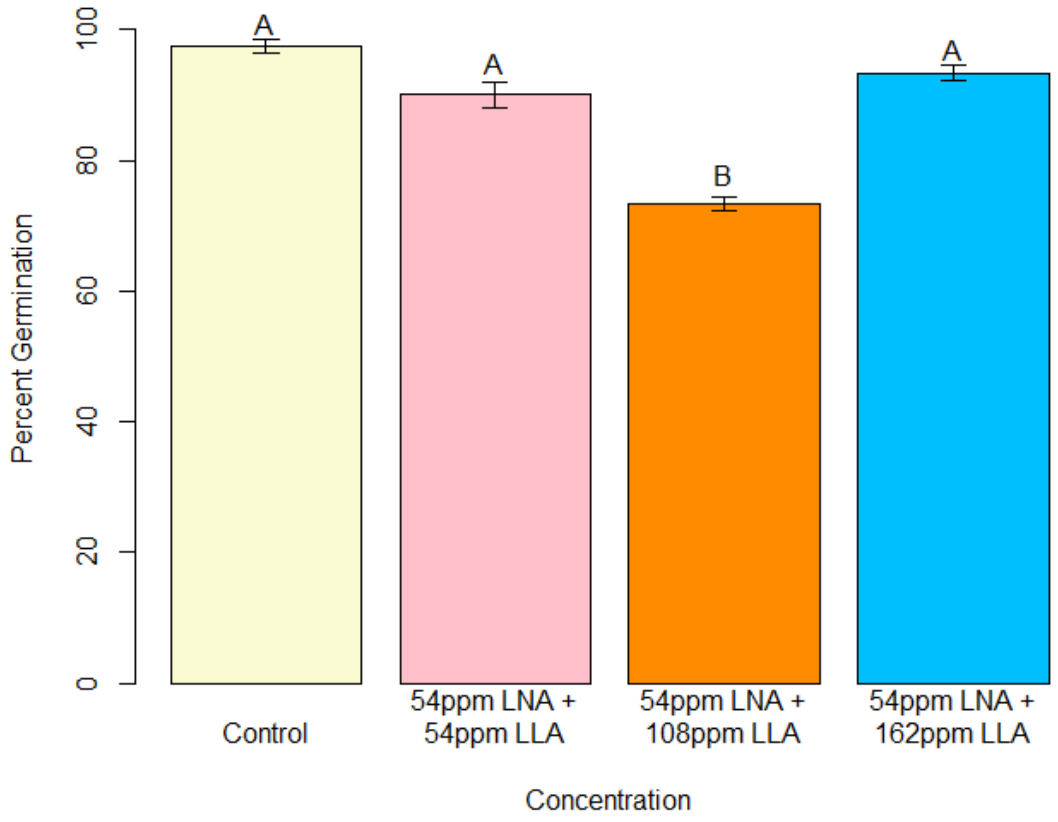

Figure 15 Percent germination of lettuce seeds 72 hours after treatment with linolenic (LNA) and linoleic (LLA) acid Mixture Two chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

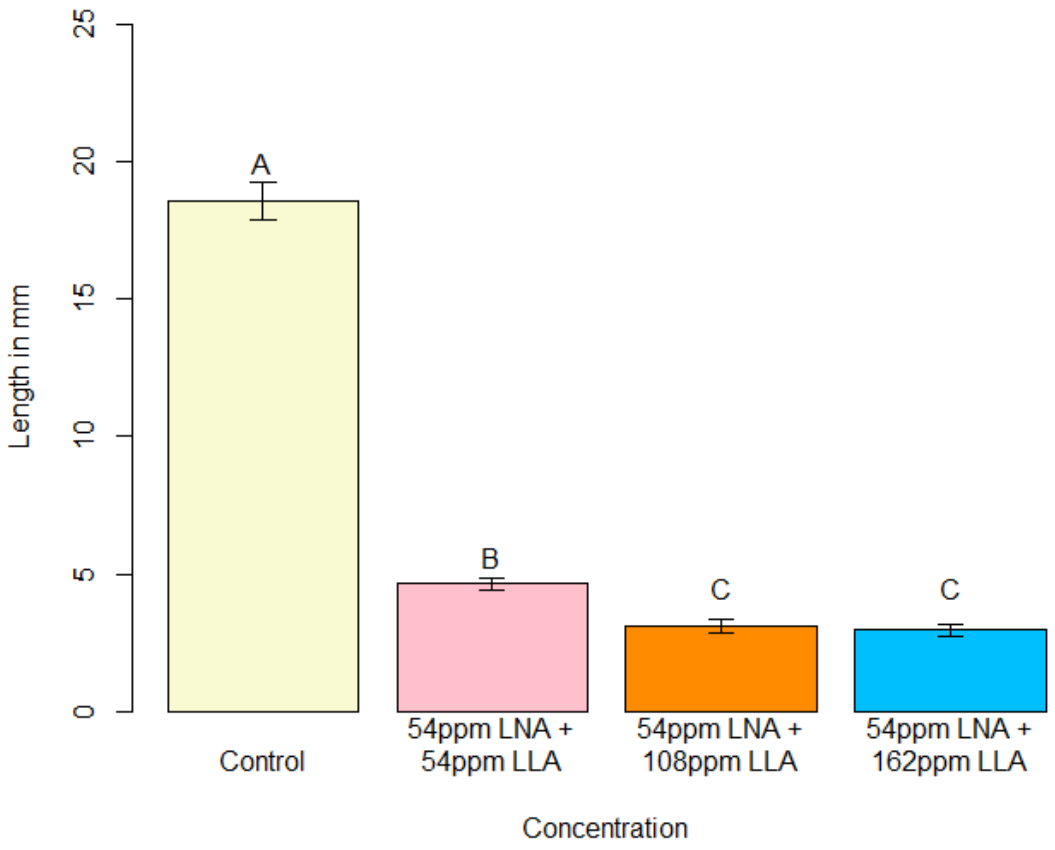

Figure 16 Germinated lettuce seedling root length 72 hours after treatment with linolenic (LNA) and linoleic (LLA) acid Mixture Two chemical solution. Differences in letters indicate a significant difference with a 0.05 confidence level. Error bars represent ±1 standard error.

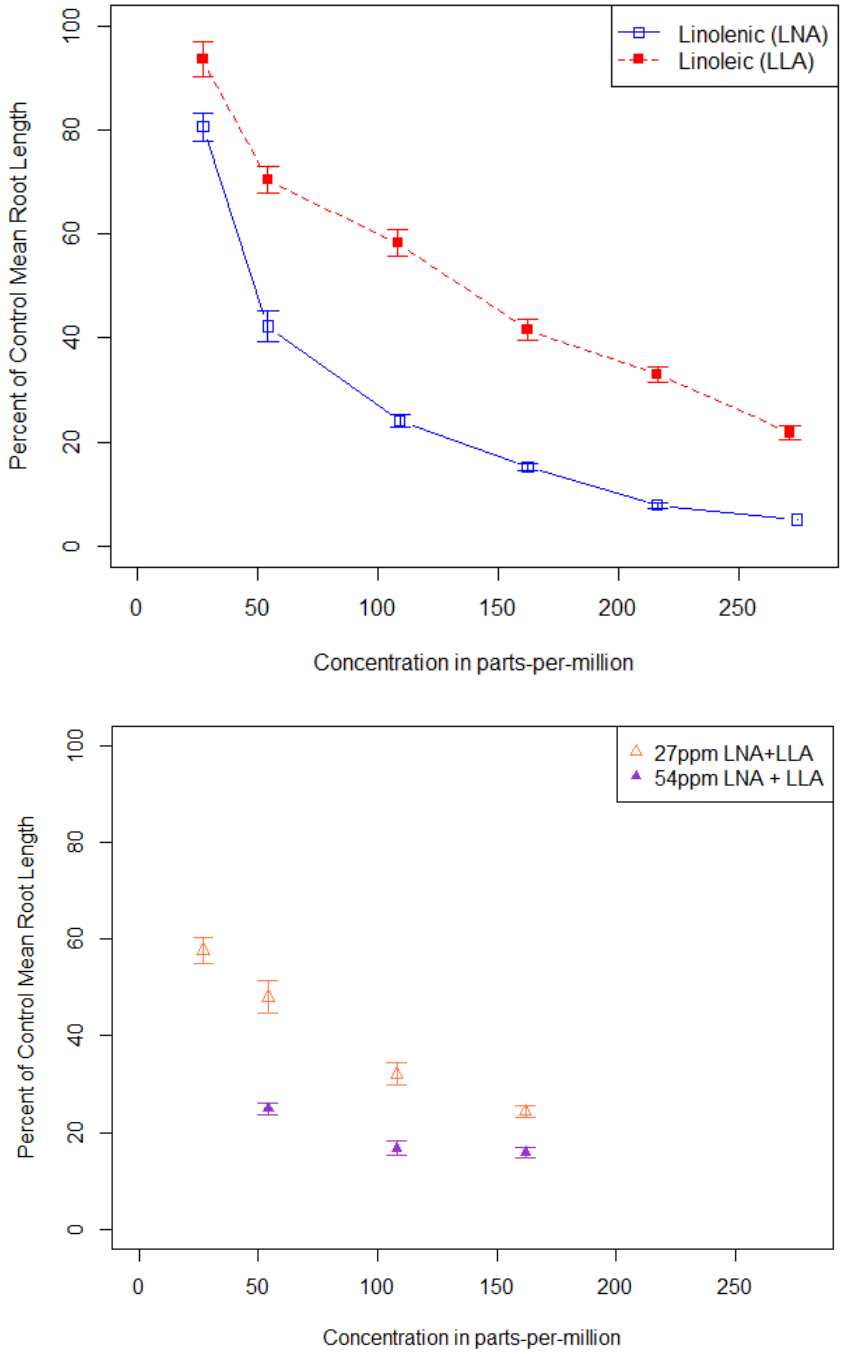

Figure 17 Lettuce seedling root length expressed as a percentage of the control for seeds germinated in different concentrations of linolenic acid (LNA) and linoleic acid (LLA)(Top image), and two different mixtures of LNA and LLA (Bottom image). Error bars represent ±1 standard error.

5.6. Biological Explanations for Reduced Growth

The process of germination should start increasing the activity of the endosperm hydrolases α-galactosidase and endo-β-mannanase (Spyropoulos and Lambiris 1980). Kontos and Spyropoulos (1996) found that application of linolenic, linoleic and jasmonic acid to ungerminated seeds of carob and fenugreek reduced the production of both of these enzymes. If lettuce seeds, or other plants affected by RCG, respond in a similar manner to fenugreek and carob seeds, it may be possible that the production of the enzymes α-galactosidase and endo-β-mannanase are hindered by the chemicals tested here.

Endo-β-mannanase is a key enzyme involved in cell wall disassembly, degradation of mannan polymers in cell walls, weakening and degradation of the endosperm, and radicle emergence (Filichkin *et al.* 2004, Buckeridge 2010). Cell wall disassembly is vital in such growth and development processes as: embryogenesis, seed germination, shoot growth, leaf formation, flower development and fruit ripening (Filichkin *et al.* 2004). Mannan polymers such as galacto-, gluco-, and galactoglucomannans are also degraded by endo-β-mannanase. This degradation then modifies the properties of cell walls (Bewley *et al.* 2000) so that growth and expansion can occur. Endo-β-mannanase has also been shown to be involved in radicle emergence by weakening of the endosperm cap (Nonogaki *et al.* 2000). While much of the research done with endo-β-mannanase has been on the tomato plant, the enzyme's activity has been well documented in lettuce seeds (Halmer *et al.* 1976, Halmer and Bewley 1979). Halmer *et al.* (1976) hypothesized that the main function of endo-β-mannanase in lettuce

seeds is likely nutrient mobilization of the endosperm cell wall polysaccharides. This provides a nutrient source for the growing embryo before it can photosynthesize. If the RCG chemicals inhibit the production of endo-β-mannanase in lettuce seeds, it is likely that this may be the mechanism of reduced seedling growth.

The other enzyme thought to be inhibited by RCG chemical application is α-galactosidase. α-galactosidase is responsible for breakdown of galactose-containing storage oligosaccharides and other polysaccharides within the seed endosperm (Dey 1981, Dey *et al.* 1983). Again, the physiological importance of this enzyme lies in its ability to mobilize polysaccharides in the beginning stages of germination. The resulting products can then be used by the growing seedling, thus supplying the initial source of energy. If nutrients cannot be mobilized the plants will likely experience reduced growth or death.

To summarize, two of the known RCG chemicals, linolenic and linoleic acid, have been shown to inhibit the production of two key enzymes. These enzymes, α-galactosidase and endo-β-mannanase, have numerous physiological functions and play key roles in nutrient mobilization for the growing seedling. Without the production of these enzymes, seedlings will likely die from lack of nutrients. This mechanism has yet to be tested with RCG, but research with other species supports the conclusion.

6. Conclusion

All three null hypotheses are rejected based on the statistical analyses of the data. The first null hypothesis is rejected based off results from the linolenic acid experiment. The second null hypothesis is rejected based off results from the palmitic, linolenic, and linoleic acid experiments. And the third null hypothesis is rejected based off comparisons between the linolenic and linoleic acid experiments.

This research documented the ability of chemicals isolated from RCG roots (linolenic, linoleic and palmitic acid) to reduce lettuce seed germination and root growth. The null hypotheses were rejected, leading to the conclusions that: (1) there is a significant difference in germination percent between the control seeds and the seeds exposed to RCG potential allelochemicals, (2) there is a significant difference in root length between the control seeds and the seeds exposed to RCG potential allelochemicals, and (3) there is a significant difference in lettuce root length amongst all three RCG potential allelochemicals. In regards to the third hypothesis, linolenic acid proved to have the strongest effect on lettuce germination and growth, followed by linoleic acid then palmitic acid.

Reed Canarygrass is now a verified allelopathic plant, some of the allelochemicals have been identified, and toxicity levels to a target species have been elucidated. Among the many questions left to still be answered about this ecologically and economically important plant are: what are the concentrations of these allelochemicals in soil with regards to distance from roots; what are the holding times of these allelochemicals in differing soils; how do these allelochemicals act in water; what are the other compounds

left to be identified in RCG, and what role does jasmonic acid and its methyl ester play in RCG? Exploring some of these questions will lead to a better understanding of RCG's ability to outcompete so many other species.

Future research with RCG could be steps in the right direction in exploring alternatives to synthetic herbicidal treatments, such as natural allelochemicals. The growing problem of worldwide herbicidal resistance brings about the need for environmentally-friendly weed management technologies (Albuquerque *et al.* 2011). Research into RCG and species alike could lead to isolation and identification of compounds that have the ability to naturally control unwanted weed species.

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8. Appendix

A. Palmitic Acid

Germination

Concentration(ppm)	germinated
0	10
0	10
0	10
100	9
100	10
100	10
200	10
200	9
200	10
500	10
500	8
500	5

Root Length

conc.	length	conc.	length	conc.	length	conc.	length
0	21	0	19	100	18	200	24
0	20	0	25	100	29	200	20
0	15	0	24	100	24	200	15
0	16	0	13	100	20	200	16
0	16	0	13	100	11	500	11
0	18	0	16	100	18	500	8
0	17	0	15	100	16	500	8
0	11	100	13	100	13	500	17
0	15	100	18	200	23	500	9
0	20	100	15	200	29	500	13
0	22	100	11	200	17	500	11
0	23	100	20	200	22		
0	23	100	15	200	21		
0	16	100	12	200	23		
0	21	100	25	200	16		
0	23	100	17	200	13		
0	17	100	20	200	22		
0	19	100	20	200	15		
0	19	100	21	200	21		
0	21	100	17	200	18		
0	20	100	15	200	16		

B. Linoleic acid
Germination

concentration(ppm)	germinated
0	10
0	10
0	10
0	9
27	10
27	10
27	8
27	10
54	8
54	10
54	8
54	10
108	10
108	8
108	10
108	8
162	8
162	8
162	10
162	10
216	10
216	10
216	10
216	8
271	7
271	9
271	7
271	9

Root Length

conc.	length	conc.	length	conc.	length	conc.	length
0	21	0	15	54	15	162	11
0	22	0	19	54	8	162	7
0	16	0	17	54	11	162	8
0	24	0	20	108	13	162	6
0	14	0	15	108	10	162	5
0	16	0	16	108	15	216	9
0	17	0	19	108	13	216	8
0	18	0	16	108	11	216	4
0	18	0	19	108	10	216	6
0	19	0	20	108	5	216	5
0	20	0	15	108	11	216	6
0	23	0	22	108	13	216	8
0	18	0	13	108	10	216	5
0	23	0	21	108	12	216	5
0	15	27	24	108	10	216	5
0	21	27	21	108	14	216	6
0	16	27	19	108	10	216	6
0	24	27	18	108	8	216	6
0	21	27	18	108	13	216	5
0	23	27	14	108	11	216	6
0	28	27	14	108	13	216	8
0	19	27	18	108	10	271	6
0	17	27	12	108	10	271	3
0	15	27	13	108	8	271	4
0	15	27	19	108	13	271	6
0	18	27	17	108	12	271	2
0	15	27	16	108	10	271	4
0	17	27	19	108	13	271	2
0	16	27	23	108	10	271	5
0	13	27	17	108	8	271	5
0	14	27	19	108	10	271	4
0	23	27	12	108	13	271	4
0	19	27	19	108	14	271	5
0	21	27	18	162	10	271	4
0	22	27	15	162	10	271	3
0	23	54	16	162	6	271	4
0	16	54	11	162	10	271	4

0	16	54	15	162	7	271	3
0	20	54	15	162	6	271	5
0	20	54	15	162	10	271	5
0	20	54	13	162	8	271	6
0	17	54	14	162	6	271	3
0	20	54	14	162	9	271	4
0	20	54	11	162	7	271	5
0	15	54	10	162	7	271	1
0	19	54	15	162	6		

C. Linolenic acid

Germination

concentration(ppm)	germinated
0	10
0	10
0	9
0	9
27	10
27	10
27	8
27	9
54	6
54	7
54	8
54	5
109	7
109	8
109	7
109	6
162	8
162	6
162	7
162	6
216	5
216	5
216	6
216	6
274	4

274	3
274	5
274	5

Root Length

conc.	length	conc.	length	conc.	length	conc.	length
0	21	0	27	54	9	162	3
0	22	0	20	54	10	162	2
0	16	0	16	54	8	162	3
0	24	0	17	54	7	162	4
0	14	27	17	54	3	162	3
0	16	27	17	54	13	216	2
0	17	27	18	54	15	216	2
0	18	27	20	54	14	216	2
0	18	27	19	54	10	216	2
0	19	27	17	54	10	216	1
0	20	27	11	109	4	216	1
0	23	27	14	109	4	216	2
0	18	27	14	109	4	216	1
0	23	27	20	109	3	216	2
0	15	27	20	109	4	216	1
0	21	27	16	109	4	216	1
0	16	27	15	109	5	274	1
0	24	27	13	109	4	274	1
0	21	27	13	109	6	274	1
0	23	27	16	109	5	274	1
0	28	27	13	109	6	274	1
0	19	27	14	109	6	274	1
0	17	27	14	109	6	274	1
0	15	27	16	109	5	274	1
0	15	27	19	109	4	274	1
0	18	27	13	109	2		
0	15	27	18	109	6		
0	17	27	19	109	7		
0	16	27	12	109	5		
0	13	54	6	109	5		
0	14	54	6	162	3		
0	22	54	6	162	4		

0	20	54	11	162	4
0	24	54	6	162	3
0	18	54	6	162	3
0	24	54	7	162	3
0	23	54	11	162	2
0	25	54	6	162	2
0	25	54	10	162	3
0	20	54	10	162	3
0	26	54	6	162	3
0	25	54	4	162	2
0	20	54	8	162	2
0	21	54	5	162	4
0	18	54	10	162	4

D. Methyl Jasmonate

Germination

concentration(ppm)	germinated
0	9
0	10
0	10
0	10
2	9
2	10
2	10
2	10
5	9
5	9
5	8
5	10

Root Length

conc.	length	conc.	length	conc.	length
0	21	0	15	2	4
0	22	0	17	2	3
0	16	0	16	2	3
0	24	0	13	2	2
0	14	0	14	5	2
0	16	2	4	5	3
0	17	2	4	5	3
0	18	2	4	5	2
0	18	2	4	5	3
0	19	2	4	5	3
0	20	2	3	5	2
0	23	2	2	5	2
0	18	2	3	5	2
0	23	2	3	5	2
0	15	2	4	5	3
0	21	2	5	5	3
0	16	2	5	5	2
0	24	2	4	5	2
0	21	2	4	5	3
0	23	2	3	5	2
0	28	2	3	5	3
0	19	2	4	5	3
0	17	2	3	5	4
0	15	2	4	5	3
0	15	2	3	5	4
0	18	2	3	5	3
				5	2

E. Mixture One Experiment

Germination

concentration(ppm)	germinated
0	10
0	10
0	10
0	9
27	10
27	8
27	9
27	10
54	8
54	10
54	10
54	8
108	10
108	10
108	9
108	9
162	9
162	10
162	10
162	9

Root Length

conc.	length	conc.	length	conc.	length	conc.	length
0	21	27	8	108	4	27	12
0	22	27	8	108	6	27	10
0	16	27	8	108	6	27	8
0	24	27	9	108	5	108	2
0	14	27	9	108	4	108	6
0	16	27	10	108	5	108	5
0	17	27	8	108	6	162	6
0	18	27	13	108	5	162	4
0	18	27	12	108	5	162	4
0	19	27	15	108	6	162	4
0	20	27	8	108	5	162	3
0	23	27	12	108	3	162	4
0	18	27	12	108	5	162	4
0	23	54	11	108	6		
0	15	54	14	108	5		
0	21	54	12	108	4		
0	16	54	12	108	6		
0	24	54	15	108	11		
0	21	54	8	108	7		
0	23	54	7	108	8		
0	28	54	11	108	10		
0	19	54	12	108	10		
0	17	54	7	108	10		
0	15	54	13	162	4		
0	15	54	12	162	5		
0	18	54	5	162	4		
0	15	54	7	162	4		
0	17	54	10	162	4		
0	16	54	10	162	4		
0	13	54	7	162	4		
0	14	54	7	162	4		
27	12	54	6	162	8		
27	15	54	6	162	6		
27	13	54	8	162	3		
27	15	54	5	162	6		
27	8	54	5	162	5		
27	11	54	4	162	5		

F. Mixture Two Experiment

Germination

concentration(ppm)	germinated
0	9
0	10
0	10
0	10
54	9
54	10
54	8
108	8
108	7
108	7
162	10
162	9
162	9

Root Length

conc.	length	conc.	length	conc.	length
0	21	54	7	54	6
0	22	54	4	54	4
0	16	54	5	54	7
0	24	54	6	54	4
0	14	54	4	54	4
0	16	54	6	54	4
0	17	54	3	54	4
0	18	54	5	162	5
0	18	54	4	162	2
0	19	108	2	162	3
0	20	108	3	162	2
0	23	108	3	162	2
0	18	108	4	162	2
0	23	108	3	162	2
0	15	108	3	162	2
0	21	108	5	162	2
0	16	108	6	162	3
0	24	108	6	162	3
0	21	108	2	162	2
0	23	108	2	162	2
0	28	108	2		
0	19	108	1		
0	17	108	1		
0	15	108	2		
0	15	108	3		
0	18	108	3		
0	15	108	3		
0	17	108	4		
0	16	108	4		
0	13	162	4		
0	14	162	4		
54	4	162	4		
54	4	162	5		
54	3	162	4		