EFFECTS OF β-ESTRADIOL ON GERMINATION AND GROWTH IN ZEA MAYS L.

A THESIS PRESENTED TO
THE DEPARTMENT OF NATURAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF MASTER OF SCIENCE

BY
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NORTHWEST MISSOURI STATE UNIVERSITY MARYVILLE, MISSOURI
2014

EFFECTS OF β-ESTRADIOL ON THE GERMINATION AND GROWTH IN ZEA MAYS L.

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Abstract

Water is one of the most important resources for an ecosystem. Pollution of major water sources has become a serious problem across much of the world. One example of water pollution caused by humans is the dumping of waste products as effluent into major rivers and waterways. While documentation for the effects of water contaminants on a variety of animals has been widely researched and documented, studies on the effects of these same contaminants on plants are relatively new. Current research has focused on the effects of these contaminants either on plant germination or on vegetative plant growth. The purpose of this research was to investigate the effects of a major pollutant, β-estradiol, on the germination and vegetative growth of corn (*Zea mays* L.). The concentrations used in these experiments were 50 μg/L, 0.1 mg/L, 1.0 mg/L and 10 mg/L. In the germination experiment, total percent germination, mean hour of germination, primary root length, coleoptile length, and number of adventitious roots was investigated. The parameters used in the growth experiment were overall root length, overall shoot length, number of leaves, and chlorophyll content. Corn kernel germination and corn seedling growth were consistently inhibited by the 10 mg concentration. The 0.1 mg treatment augmented germination and seedling growth. Future experiments could be carried out to follow the development of the corn seedlings through maturation and the production of fruit to determine if the high doses of β-estradiol affect the later stages of development as well.

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Chapter 1: Introduction

Ecosystems consist of complex and intricate relationships between organisms and their biotic and abiotic surroundings. Each species in an ecosystem occupies a niche, and often their role in the community is vital to the functionality of it. When there is a change to any part of the biotic or abiotic resources in an ecosystem, it can be drastically altered (Islam and Tanaka 2004). One of the most important resources for an ecosystem is water. As a key reactant in photosynthesis, water helps to form the base of any ecosystem by providing the necessary resources for photosynthetic organisms, which then in turn provide sustenance for heterotrophic organisms as well as oxygen for aerobic organisms (Campbell et al. 2011). The sum of all of these organisms and the flow of energy from one trophic level to the next creates an intricate and balanced chain or web. If disturbed or altered, the flow of energy can become highly disrupted causing collapse of the entire ecosystem (Campbell et al. 2011). As water helps form the base of these intricate relationships, any alteration of the water supplied to an ecosystem is likely to have drastic effects on the organisms that live there.

Properties of Water

Water contains unique properties that allow it to sustain life on this planet. The molecule itself, made of two hydrogen atoms bonded to one oxygen atom, is held together by covalent bonds. These bonds are considered to be polar due to the high electronegativity, or stronger pull on the shared electrons, of the oxygen atom in comparison to the hydrogen (Campbell et al. 2011). The polar covalent bonds between

the atoms create a molecule that has a partial negative and a partial positive pole due to the negatively charged electrons spending the majority of their time around the oxygen atom. When the molecules are next to each other they align themselves so that the negative pole of one water molecule is associated with the positive pole of another. These create hydrogen bonds between water molecules. While one hydrogen bond by itself is rather weak, the accumulation of many hydrogen bonds creates an extremely strong collective force that ends up giving water its unique properties that allow it to sustain life as we know it (Campbell et al. 2011).

One of these properties is the ability of water to hydrogen bond with itself and create a high surface tension. This property is known as cohesion and is the result of many hydrogen bonds working together. Cohesion becomes an important part of the process by which plants will move water into the root tissue and up through the vascular tissue. The process by which water is transported through a plant's tissues is known as adhesion-cohesion theory of water movement (Taiz and Zieger 2006). The driving force behind this is transpiration. This involves the evaporation of water through openings in the leaves, known as stomata. The hydrogen bonds between the molecules create a chain of water that gets pulled through the xylem tissue of the root, up the stem and ultimately evaporates into the atmosphere through the stomata (Campbell et al. 2011). Adhesion, or the ability of water molecules to cling to other surfaces, also plays a role as it allows the water molecules to adhere to the cell walls of xylem tissue and help counter the effects of gravity (Campbell et al. 2011).

Another life supporting property of water is its ability to be a universal solvent for almost any other polar molecule. This property allows water to dissolve many different substances, and then transport and cycle them through organisms' tissues as well as through the environment (Campbell et al. 2011). There can be quite severe consequences to organisms because of the solvent properties of water, as it is not a selective process. Several toxic and synthetic substances that are polar could be dissolved in water and consequently taken up into the organism's tissues. Potentially, these substances can then accumulate and cause damage or harm to the organism (D'Abrosca et al. 2008).

Water pollution

One example of water pollution caused by humans is dumping waste products as effluent into major rivers and waterways. Although this practice has occurred for centuries, the damaging environmental effects have only recently been brought to light by the research that is being conducted by scientists worldwide on various organisms across most kingdoms and phyla (Islam and Tanaka 2004). As rivers take their course, ecosystems downstream are affected. As these waterways flow into the oceans through estuaries many coastal and marine ecosystems are now being impacted as well. To date, the greatest amount of waste that ends up in marine ecosystems is sewage (Islam and Tanaka 2004). Sewage effluent can include industrial waste, municipal waste, animal remains, slaughterhouse wastes, water and wastes from domestic baths, kitchen

wastes, fecal matter and numerous other things (Islam and Tanaka 2004). For a population of 800,000 people it has been estimated that approximately 3600 tons of organic matter are dumped annually (Islam and Tanaka 2004). With worldwide populations now approaching 8 billion people, the amount of organic waste ending up in aquatic ecosystems is astronomical.

Endocrine Disruptors

Sometimes naturally occurring chemicals, such as hormones, can also build up in these aquatic environments due to waste effluent. One specific class of these is called reproductive endocrine disruptors, or REDs. Several studies have been conducted that show where there are high levels of REDs in habitats there tends to be disruptions in the morphology and physiology of the organisms that reside there (Islam and Tanaka 2004). Examples of synthetic and naturally occurring chemicals that act as REDs include estrogenic and anti-androgenic substances. There are also chemicals that are not hormonal in nature but can have estrogenic properties, such as alkyphenols, industrial pesticides, and chlorinated hydrocarbons (Islam and Tanaka 2004). One of the most common estrogenic sources in waste water effluent comes from the urine of women who take birth control pills. These hormone treatments contain the synthetic ethynylestradiol as well as significant amounts of 17β-estradiol (Figure 1.1) (Batty and Lim 1999).

The study of the effects of growing concentrations of these chemicals known as REDs in aquatic ecosystems is extremely important due to the fact that there is a large

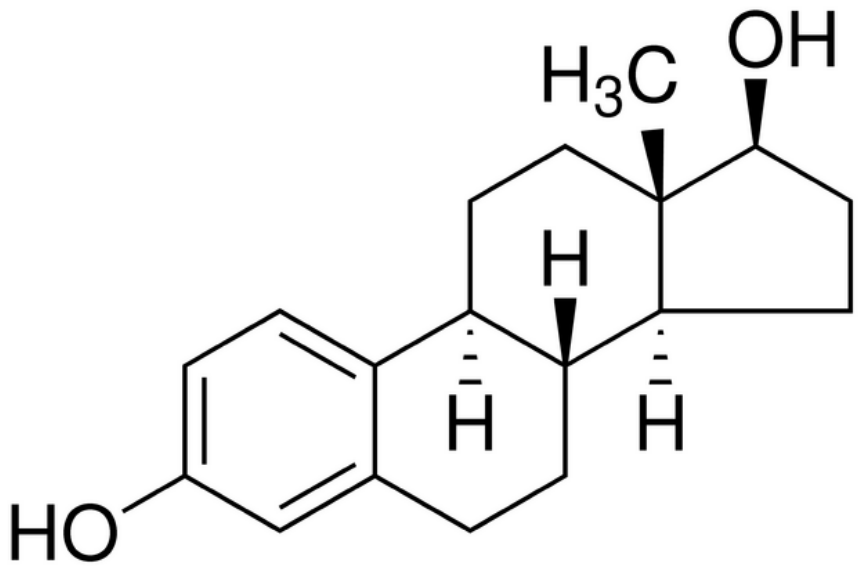


Figure 1.1. The molecular structure of the mammalian sex hormone 17β-estradiol.

number of organisms that show hormone-dependent sexual dimorphism, or hormonal differences that are present in the development of males versus females. Reproductive disorders in wildlife can include anything from altered fertility and reduced offspring viability to impaired hormone functionality and modified reproductive anatomy (Guillette et al. 2000). The most common disruption caused from REDs is due to estrogen mimicry. These contaminants have the ability to induce cellular proliferation of estrogen sensitive cells (such as breast and ovarian tissue) as well as bind to estrogen receptors (Guillette et al. 2000).

A study was conducted on the mosquitofish, *Gambusia affinis holbrooki*, to determine if there was an effect on the organisms from polluted aquatic environments (Batty and Lim 1999). In this study fish were collected from multiple sections of the same river. One of the sites was located upstream of a sewage/waste water treatment facility and another was located downstream. This mosquitofish is one example of an organism that exhibits hormone-dependent sexual dimorphism. The males have a modified anal fin called the gonopodium that is used to aid in sperm packet transfer to females (Batty and Lim 1999). The elongation and development of the gonopodium is under androgenic control. Thus, when the researchers found that gonopodium length and overall number of male fish present was significantly reduced in populations caught downstream of the sewage effluent, there was evidence to support the hypothesis that REDs are having an impact on wildlife (Batty and Lim 1999). These fish were originally introduced to help keep mosquito populations at bay. They have since become an integral part of the ecosystem by also providing food for other species of aquatic

organisms. By reducing their reproductive success, their numbers are starting to dwindle downstream of the sewage effluent which in turn will start to impact other organisms dependent on them (if they themselves are not already directly impacted by the effluent).

Another organism that is exhibiting the effects of REDs is *Alligator mississippiensis,* or the American alligator. Studies of populations in polluted Florida lakes have shown that juvenile alligators have different plasma concentrations of hormones along with altered reproductive tract anatomy and hepatic functioning compared to juvenile alligators from non-polluted lakes nearby (Guillette et al. 2000). It was also noted that clutch viability, or the survival rate of the eggs, was greatly reduced in the polluted environments. Several alterations in the reproductive tract were noted in hatchlings and persisted throughout the life of an alligator. More specifically, it was noted that males had reduced phallus size as well as lower plasma levels of testosterone than normal. This would indicate that the REDs are affecting the development of the alligators starting when they are still an embryo (Guillette et al. 2000). This poses a serious problem for appropriate alligator growth as the developing embryo shows extreme sensitivity to chemical signals.

While documentation for the effects of water contaminants on a variety of animals has been widely researched and documented, studies on the effects of these same contaminants on plants are relatively new. Mammalian sex hormones were originally found in plants and documented in 1926 by Dohrn et al. (Erdal et al. 2010). Between the original discovery and up until the 1990's the presence of the hormones

was determined in 128 plant species from 50 families but the effects of the hormones were not studied in great detail (Janeczko and Skoczowski 2005). With the large environmental movement that ushered in the millennium, scientists started to take another look at the role that contaminants play in the environment and especially started to look at their effects on plants. Current research has focused on the effects of these contaminants either on plant germination or on vegetative plant growth (D'Abrosca et al. 2008).

Estradiol

A potent mammalian sex hormone produced primarily in the ovaries that is derived from cholesterol is 17β-Estradiol (Carreau et al. 2002). The chemical formula of 17β-Estradiol is $C_{18}H_{24}O_2$ and it has a molecular weight of 272.38 grams per mol (Figure 1.1). The steroid can freely enter an animal cell and when bound to a ligand has the ability to enter the nucleus; here it is involved with regulating gene transcription. In female mammals this steroid acts as a growth hormone for specific tissues in reproductive organs such as breast tissue, and is also responsible for maintaining the oocytes in the ovaries (Carreau et al. 2002). Estradiol also exerts wide and varying effects on other tissues in the body such as the ability to change the shape of bone and joints, as well as affect fat deposition and skin composition.

To treat cases of hypoestrogenism, or low estrogen levels, medication containing 17β-Estradiol is often given to women. It is also one of the main chemical compounds (or derivatives of 17β-Estradiol) in most oral birth control pills (Carreau et al. 2002).

Mammals naturally excrete 17β-Estradiol through sweat glands and in their urine, but increased consumption through synthetic methods can lead to increased excretion. These excretions then end up in the effluent of our waste water and are carried to waste water treatment facilities. The environmental protection agency (EPA) has listed 17β-Estradiol as an unregulated drinking water contaminate on their Contaminate Candidate List 3 (CCL3). Contaminates that are found on CCL3 are not subject to any national primary drinking water regulations (United States Environmental Protection Agency, 2012). The Safe Drinking Water Act (SDWA) of the United States originally passed by Congress in 1974 (amended in 1986 and 1996) ensures that Americans have access to quality drinking water and that actions are taken to protect drinking water and its sources. The contaminants listed on the CCL3 are those that are toxic enough that, under the SWDA, may require regulations in the future (United States Environmental Protection Agency, 2012).

Seed Germination

Seed germination is a complex physiological process that includes the action of multiple processes. Plant hormones, water relations, light responses, temperature, and stimulation of the expression of genes all have a part in regulating germination. Once all of the conditions have been met a seed will begin to germinate, or leave the dormant stage and continue growth (Taiz and Zeiger, 2006). The entire process of germination will transform the embryonic plant into a seedling. In order for growth of the embryo to continue the cells will require energy in the form of ATP provided by the process of

cellular respiration. Respiration requires glucose as well as oxygen. In order to obtain high oxygen levels the seed coat must be penetrated (Taiz and Zeiger, 2006). In most plants this occurs when the seed imbibes, or uptakes, large quantities of water. This causes the tissues to swell, cracking the seed coat in the process.

Ions and molecules in the water are taken up into the tissues of the seed as well. Compounds contained in the water could have an effect on the germination of the seed. Studies across both Europe and North America have shown that surface water, ground water, and drinking water systems contain contaminants (D'Abrosca et al. 2008). Studies conducted on varying species of plants demonstrated that β-estradiol decreased the germination percentage of lettuce, carrot, and tomato seeds (*Lactuca sativa* L., *Daucus carota* L., and *Lycopersicon esculentum* Mill. respectively). Germination was reduced by 57% in *L. sativa*, 6% in *D. carota*, and 18% in *L. esculentum* when compared to the controls (D'Abrosca et al. 2008). In the same study the germinated seedlings were collected and the root length was measured. Seedlings of two species exhibited marked reduction in root length when compared to the control. There was a 34% reduction in *L. sativa* and 22% reduction in *L. esculentum.* However, *D. carota* actually exhibited an 11% increase in overall root length (D'Abrosca et al. 2008).

Another study performed on chickpea seeds (*Cicer arietinum* L.) resulted in a dramatically different set of results. Both β-estradiol and progesterone increased seed germination. After 48 hours seed germination increased from 85% in the control to 100% in the β-estradiol treated group (Erdal and Dumlupinar, 2010). Subsequently,

these treated seedlings also demonstrated a significant amplification to both the root length and the shoot length. Yet another study performed on wheat (*Triticum aestivum* L.) showed that there was no significant effect on seed germination (Nirmala et al. 2008).

Importance of Grain Crops

Farmland across the Midwest portion of the United States is typically dominated by soybeans (*Glycine max* (L.) Merr.) or field corn (*Zea mays* L.). Corn (or maize) is primary grown for livestock feed and silage, although a significant percentage is now being used in the production of ethanol. One bushel of corn will provide 10.6 L of ethanol, 7.7 kg of dried distillers grain, 25.4 kg of grain and 14.5 kg of starch or 14.9 kg of sweetener or .73 kg of corn oil (University of Missouri Extension, 2012). The economic value of corn in the U.S. (Fig 1.2) is at an all-time high and was recorded to be around two billion dollars in 2010 (University of Missouri Extension, 2012). Understanding the possible effects of environmental pollutants on corn production and yield is therefore one of great consequence to farmers and the American economy. The purpose of the experiments in subsequent chapters was to determine if a contaminate selected from the CCL3 list would have an effect on the germination or growth of corn. The contaminant chosen was 17β-Estradiol. One of the reasons for this decision was the close association between farmland and large livestock operations. 17β-Estradiol can be found in significant quantities in both the excrement and urine of the livestock in these operations, and often works its way into the water systems and also onto fields (Caron et al. 2012).

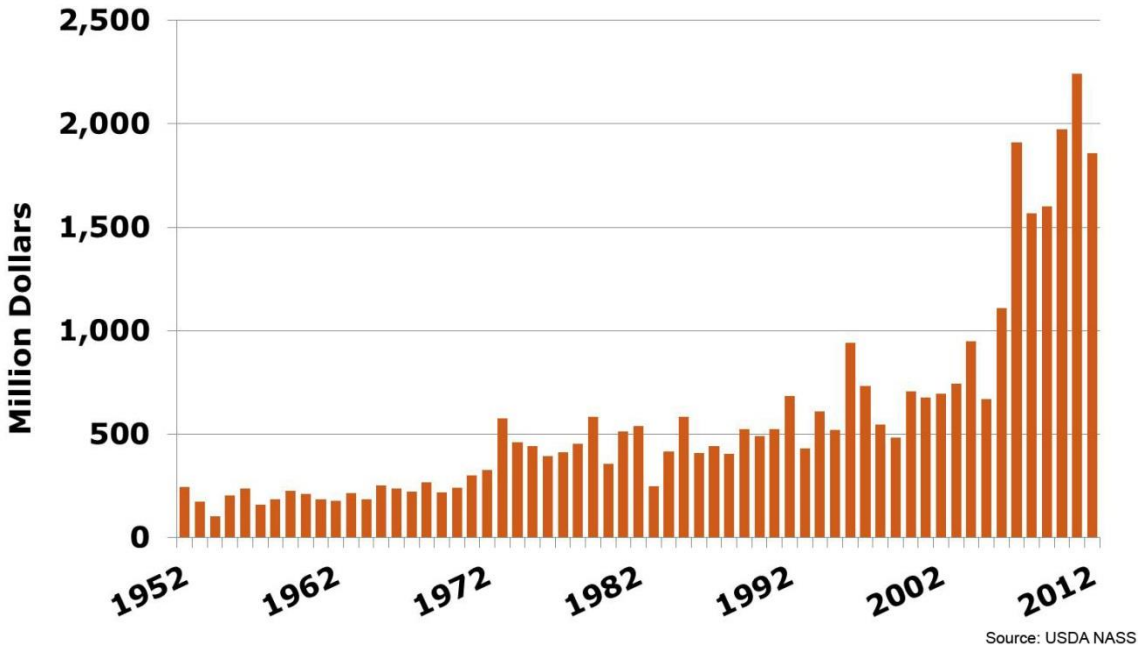


Figure 1.2. Value of corn production in the United States for the years 1952 to 2012 according to the United States Department of Agriculture (University of Missouri Extension, 2012).

Chapter 2: The effect of β-estradiol on germination of corn (*Zea mays* L.)

As previously stated in the introductory chapter, hormones such as β-estradiol can significantly affect the growth and physiology of both animals and plants. Investigations previously conducted on plants have looked at the effect of β-estradiol on the seed germination in a wide variety of different plant species. β-estradiol decreased the germination percentage of lettuce, carrot, and tomato seeds (*Lactuca sativa* L., *Daucus carota* L., and *Lycopersicon esculentum* Mill. respectively), while in chickpea seeds (*Cicer arietinum* L.) the germination percentage was increased. The variations in the results of studies suggests that the effect of the hormone will depend on the species of plant exposed to the hormone.

Corn kernels are actually fruits. Each kernel is technically classified as a caryopsis, as the simple seed inside is surrounded by the pericarp, the mature ovary wall. Fruits classified as caryopsis are also known as grains. In these fruits the seed coat is fused to the pericarp (Evert and Eichhorn 2013). The corn embryo contains a well-established embryonic axis. The radicle (root) is surrounded by the coleohriza (Fig 2.1) and the epicotyl (shoot) is enclosed by the coleoptile. The axis is attached to a single cotyledon, also known as the scutellum. Surrounding the cotyledon is the endosperm, which serves as food and energy storage that will be utilized in germination (Evert and Eichhorn 2013).

In order to germinate the kernel must break dormancy. This often requires a number of different things including proper temperature, plant hormones, water

relations, light responses, and stimulation of the expression of genes. In maize kernels the first structure to start to grow once dormancy has been broken is the coleorhiza (Evert and Eichhorn 2013). The coleorhiza emerges through the pericarp of the kernel, which is the mature ovary wall. Within the coleorhiza (Fig 2.1), the primary root starts to swiftly grow and elongate, eventually exiting through the coleorhiza (Evert and Eichhorn 2013). Once the primary root has fully emerged, the coleoptile starts to elongate and push upwards and ceases elongation once the surface of the soil is reached. At this point in germination the first leaves will start to emerge from the coleoptile. The last portion of germination occurs when adventitious roots begin to emerge from the axis where the coleorhiza (Fig 2.1) and coleoptile meet (Evert and Eichhorn 2013).

Given the economic value and importance of this grain crop and the Midwest location of this research, corn (*Zea mays* L.) was the plant selected for this experiment. Previously, to the best of my knowledge, no one had looked at the effect of β-estradiol on the germination of corn and there were no publications prior to the start of this research in 2009. The goal of this experiment was to determine if β-estradiol had an effect on the germination, primary root length, coleoptile length, and the number of adventitious roots of *Zea mays* L.

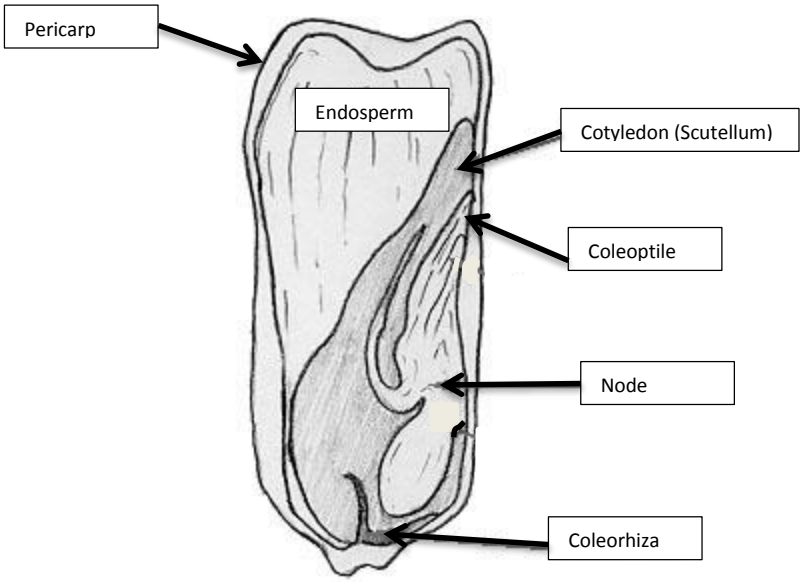


Figure 2.1. Maure corn (*Zea mays* L.) kernel.

Materials and Methods

Corn kernels (*Zea mays* L.)(Croplan Genetics Research Plot Seed L78X3068 MF113, Land O'Lakes Ag Services, Fort Dodge, IA, USA) (Fig 2.2) were surface sterilized in a 10% household bleach solution (Clorox Bleach) for 20 minutes at room temperature, and then rinsed several times with sterile deionized water. Kernels were then placed randomly into groups of ten and using a random number generator assigned to one of 25 petri dishes (Fig 2.3) lined with filter paper. Ten mg of β-estradiol (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in 1 mL of 95% ethanol and diluted with sterile water to create concentrations of 10 mg/L, 1.0 mg/L, 0.1 mg/L and 50 µg/L. Each of the 25 petri dishes was then randomly assigned to one of these four concentrations or a control (sterile water). Fifteen mL of the assigned concentration was added to each dish. Petri dishes were then placed into an incubator (Thermo Scientific model 818) without light and incubated at 26 ^{o}C (Fig 2.4). After 24 hours, the dishes were surveyed for kernels that had germinated. All germinated kernels were removed from petri dishes and placed into individual 15 ml test tubes in which a small circular piece of filter paper had been placed at the bottom along with 100 µL of β-estradiol at the assigned concentration (Fig 2.5). Germination was determined to be when the primary root began exiting the coleorhiza (Fig 2.6). Test tubes were then placed back into the incubator and kernels were allowed to grow for an additional five days. Petri dishes were also checked at 48, 72, and 96 hours and each time any kernels germinated they were put into test tubes as described above and placed back into the incubator for an additional five days.

Figure 2.2. Kernels used in this experiment were obtained from Croplan Genetics Research Plot Seed L78X3068 MF113, Land O'Lakes Ag Services, Fort Dodge, IA, USA.

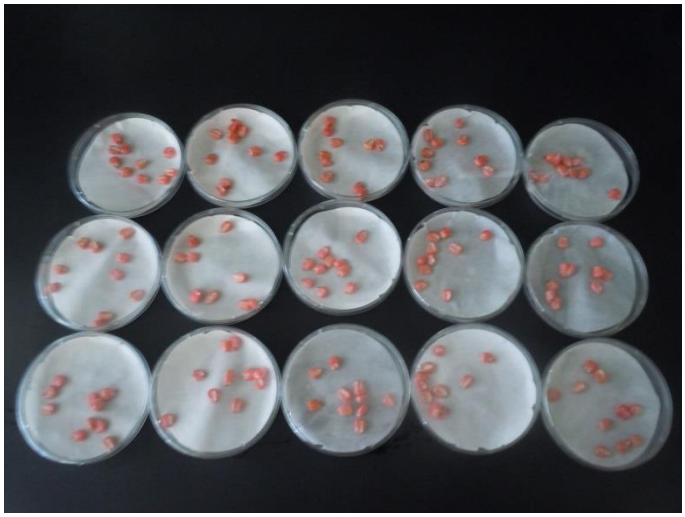


Figure 2.3. Ten corn (*Zea mays* L.) kernels were placed into petri dishes lined with filter paper. The dishes were then randomly assigned a treatment group of various β-estradiol concentrations (10 mg/L, 1.0 mg/L, 0.1 mg/L or 50 µg/L) or control.

Figure 2.4. Petri dishes containing 10 corn (*Zea mays* L.) kernels and various concentrations of β-estradiol were placed into a Thermo Scientific incubator (model 818) at 26^{o}C without light.

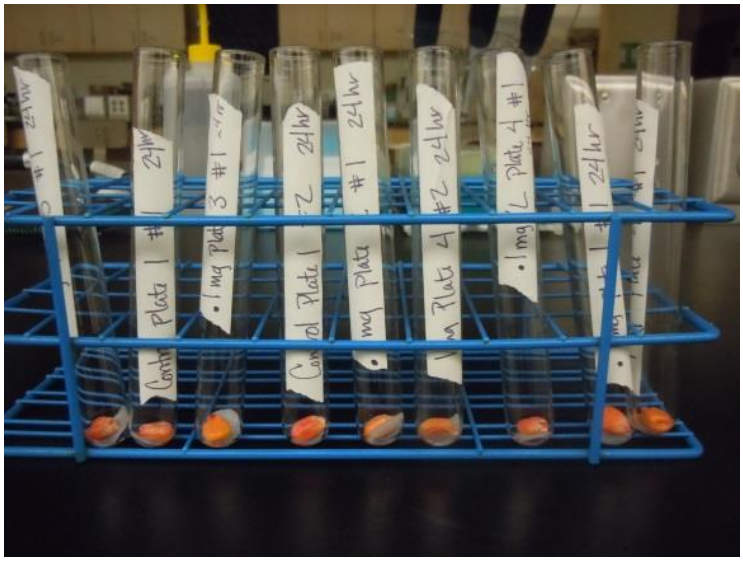


Figure 2.5. Fifteen mL test tubes in which each contained a germinated corn (*Zea mays* L.) kernel. Each kernel rests upon a piece of filter paper soaked in 100 µL of β-estradiol at the assigned concentration.

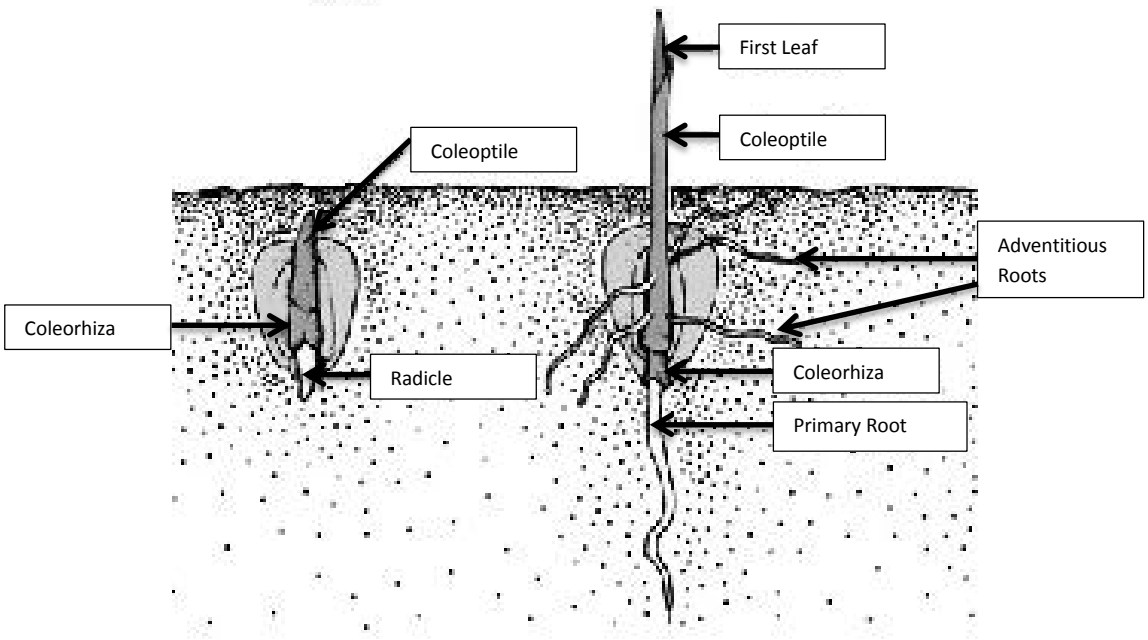


Figure 2.6. Diagram illustrating the parts of a corn (*Zea mays* L.) kernel before (left) and after (right) germination. Germination was determined to be when the primary root began to emerge from the coleorhiza. After five days of growth both the coleoptile and the primary root were removed and measured (mm). The number of adventitious roots were also counted and recorded.

At 120 hours no additional germination was recorded and the remaining kernels were discarded. Records of the number of kernels per day that germinated were kept. After five days incubation, the seedlings were removed from test tubes (Fig 2.7) and three parameters were evaluated. Lengths of the coleoptile and primary root were measured (Fig 2.6) and the number of adventitious roots counted and recorded. Each coleoptile was removed with a scalpel (Fig 2.8) and placed into a container with all other coleoptiles from the same estradiol concentration and stored at -80 ^{o}C. A similar procedure was completed for the roots. Procedures were repeated twice for two sets of 25 dishes.

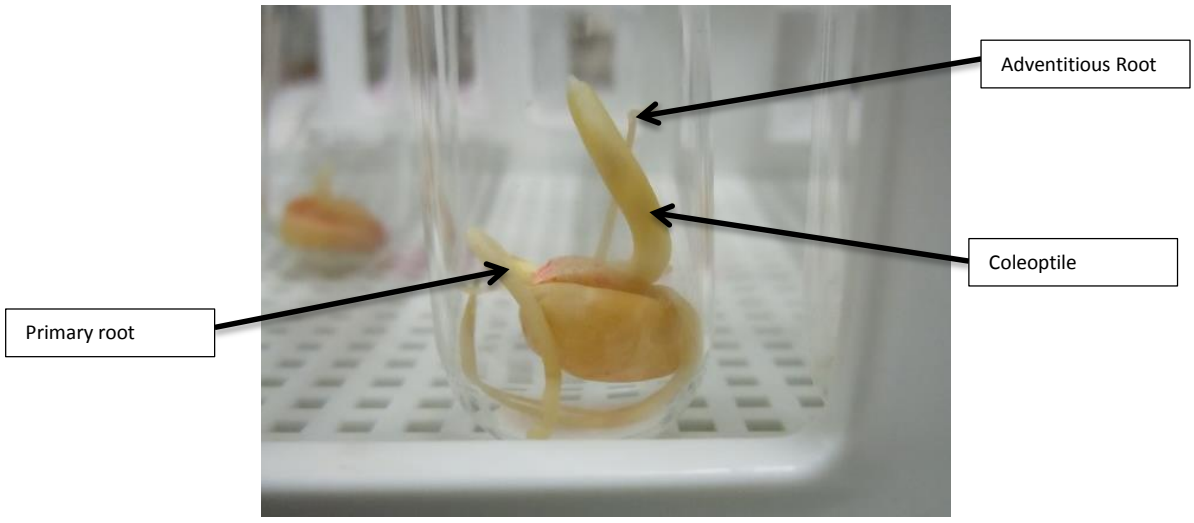


Figure 2.7. Corn (*Zea mays* L.) seedling after five days of growth post germination. The seedlings were grown in 15 mL test tubes in a Thermo Scientific incubator (model 818) without light at 26 ^{o}C.

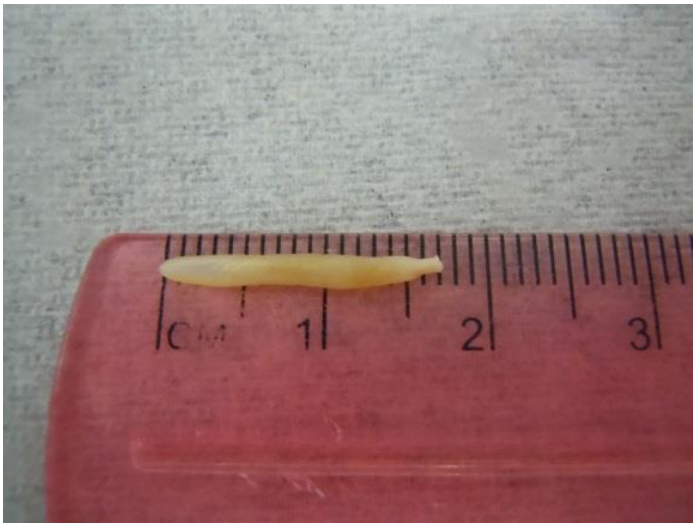


Figure 2.8. The coleoptile from a corn (*Zea mays* L.) seedling grown in the presence of 17β-estradiol for five days was removed and measured for length (mm).

Results

There were 500 corn (*Zea mays* L.) kernels used to complete this experiment but only 444 germinated, thus giving 444 observations for the analysis. It was observed that the kernels in the 10 mg β-estradiol treatment group consistently exhibited a delay in germinating. There were never kernels that germinated in 24 hours. For most treatments 48 hours was the optimal germination time, yet for the 10 mg group it was 72 hours. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was a significant difference in the amount of time it took the kernels to germinate based on the treatment the kernels were subjected to. A p-value of <.0001 was determined using a confidence interval of 97.5%, indicating that at least one of the treatments was different than the others. Using a Tukey's Studentized Range test for the hour of germination based on treatment and comparisons significant at alpha 0.025, the 10 mg treatment was computed to be significantly different from all other treatments (Fig 2.9). The 0.1 mg treatment also tested significantly different from both the control and the 50 µg treatment. The mean number of hours (Figure 2.8) for the control to germinate was 54.00 hours with a standard error of 1.47, 54.13 hours for 50 µg with a standard error of 1.34, 48.00 hours for 0.1 mg with a standard error of 1.06, 52.43 hours for 1.0 mg with a standard error of 1.43, and 66.08 hours for the 10 mg treatment with a standard error of 2.08. The treatment with the fastest germination time was determined to be the 0.1 mg treatment whereas the slowest germination was determined to be the 10 mg treatment. The difference between the means of the 0.1 mg and the 10 mg treatments is 18.08 hours.

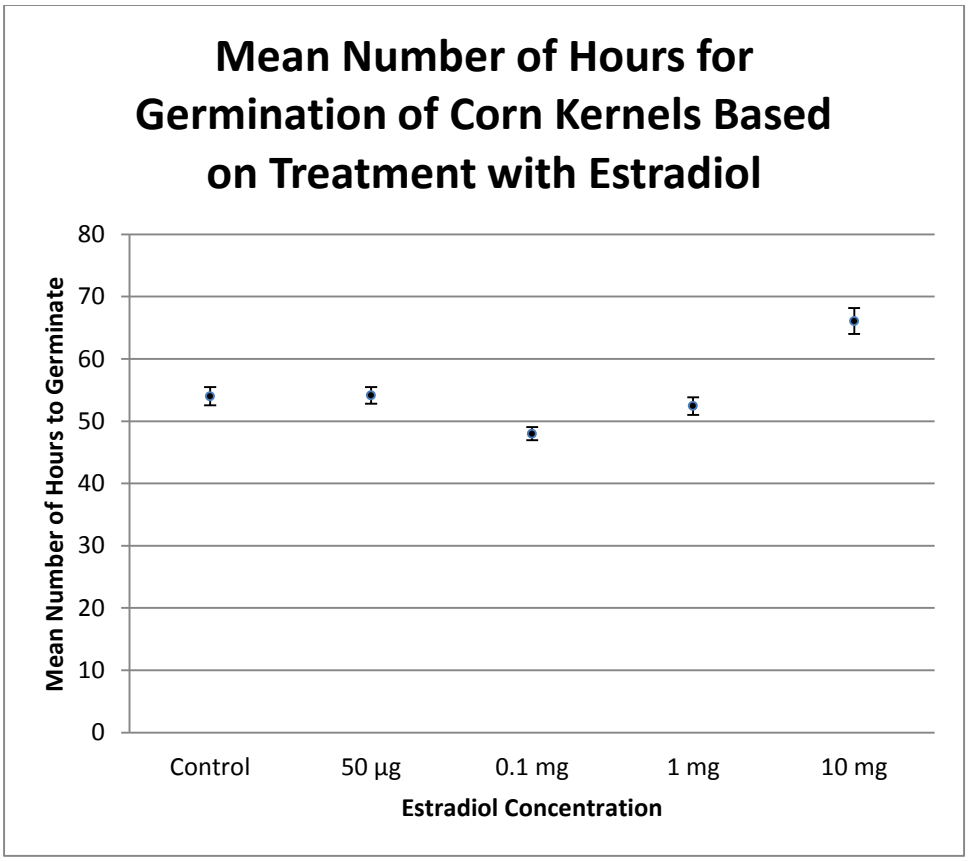
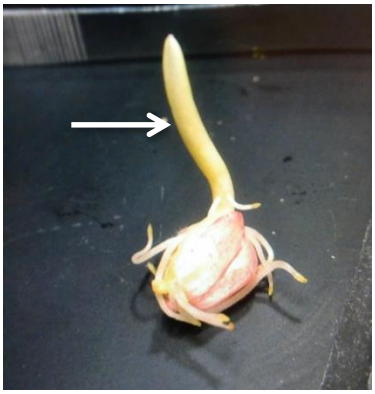


Figure 2.9. The mean number of hours for 444 corn kernels (*Zea mays* L.) subjected to different β-estradiol concentrations to germinate. Bars denote a 97.5% confidence interval.

It was observed that the kernels in the 10 mg treatment group were almost all lacking primary root growth (Fig 2.11). Most of the kernels had coleoptile growth and adventitious roots present, but the primary roots were highly undeveloped. The 10 mg group was the only one to exhibit these properties. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was a significant difference in the length of the primary root after five days of growth among the various treatment groups. Using a confidence interval of 97.5% a p-value of <.0001 was determined, indicating that at least one of the treatments was significantly different than the others. Using a Tukey's Studentized Range test for mean primary root length based on treatment and comparisons significant at alpha 0.025, it was determined that the 10 mg treatment was statistically different from every other treatment. None of the other treatments (50 µg, 0.1 mg, 1.0 mg, or control) were significantly different from each other (Fig 2.12). The mean primary root length (Fig 2.10) was 31.75 mm with a standard error of 1.35 for the control, 29.13 mm with a standard error of 1.27 for the 50 µg treatment, 29.49 with a standard error of 1.30 for the 0.1 mg treatment, 29.40 mm with a standard error of 1.48 for the 1.0 mg treatment, and 5.83 mm with a standard error of 0.78 for the 10 mg treatment. The longest mean primary root length was recorded in the control treatment group and the shortest mean primary root length was recorded in the 10 mg treatment group. The difference between the means was 25.91 mm.



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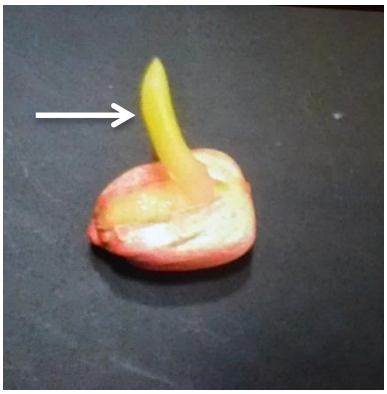

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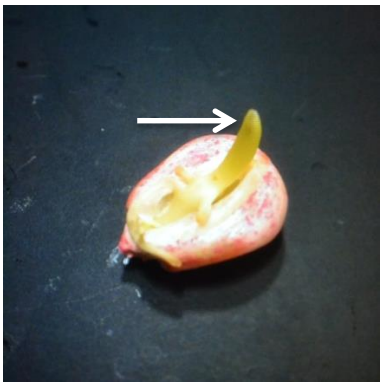

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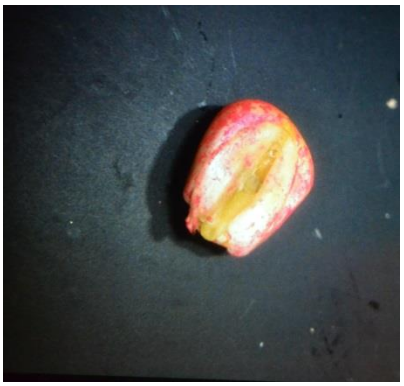

D.

Figure 2.10. Corn (*Zea mays* L.) kernels from the 10 mg β-estradiol treatment group after five days of growth with noticeable lack of primary root growth while exhibiting coleoptile (→) growth (A, B, C). A kernel from the 10 mg treatment group that germinated then proceeded to degenerate and die during the five days of growth in the 15 mL test tube (D).

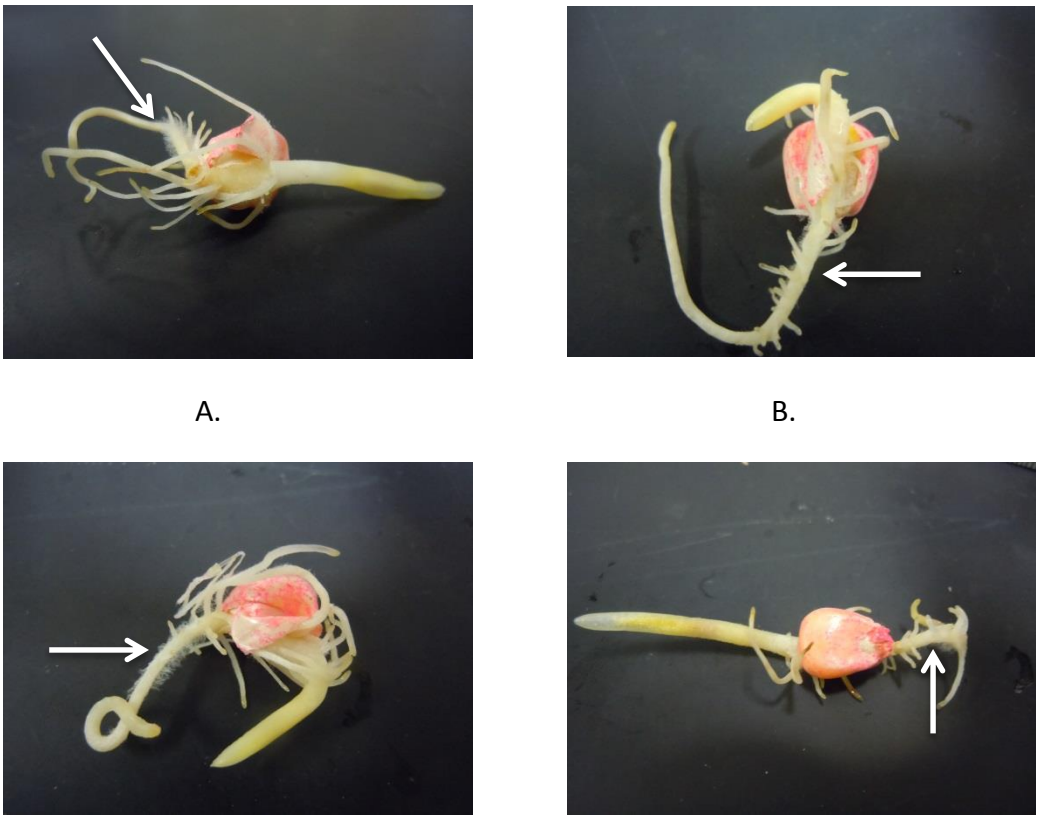

Figure 2.11. Corn (*Zea mays* L.) kernels germinated in the presence of various concentrations of β-estradiol (A,B,C). Kernel from the 1.0 mg β-estradiol treatment group (A). Kernel from the 0.1 mg treatment group (B). Kernel from the 50 µg treatment group (C). Kernel from the control group (D). Note that all kernels have a primary root (→).

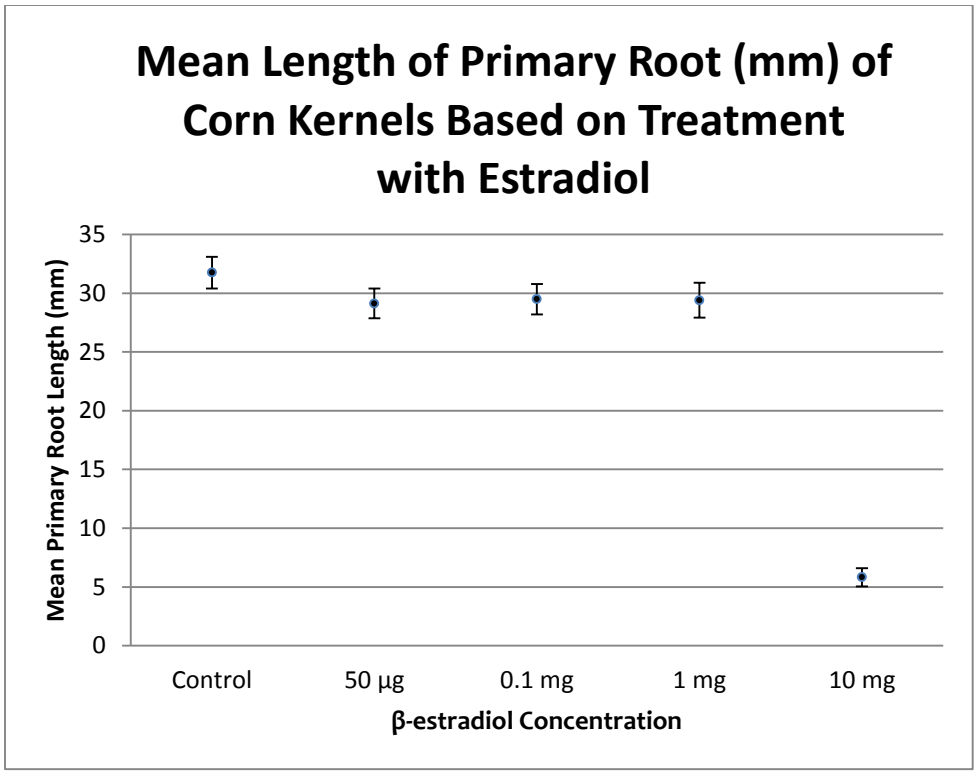

Figure 2.12. The mean primary root length (mm) of 5 day old corn (*Zea mays* L.) seedlings germinated and grown in different concentrations of β-estradiol. Vertical bars are based on a 97.5% confidence interval.

It appeared that most of the germinated kernels across the different concentrations of β-estradiol had coleoptiles that were relatively similar in both length and girth. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was a significant difference in the length of the coleoptile. Using a confidence interval of 97.5% a p-value of <.0001 was determined, indicating that at least one of the treatments was significantly different than the others. Using a Tukey's Studentized Range test for mean coleoptile length based on treatment and comparisons significant at alpha 0.025, it was determined that the 10 mg treatment was statistically different from every other treatment except for the 50 μg treatment. None of the other treatments (0.1 mg, 1.0 mg, or control) were significantly different from each other as well as the 50 μg did not test significant against the other treatments. The mean coleoptile length (Figure 2.13) was 15.20 mm with a standard error of 0.70 for the control, 13.54 mm with a standard error of 0.75 for the 50 μg treatment, 15.82 mm with a standard error of 0.74 for the 0.1 mg treatment, 15.67 mm with a standard error of 0.78 for the 1.0 mg treatment, and 10.95 mm with a standard error of 0.77 for the 10 mg treatment. The longest mean coleoptile length was recorded in the 0.1 mg treatment group and the shortest mean coleoptile length was recorded in the 10 mg treatment group. The difference between the means was 4.87 mm.

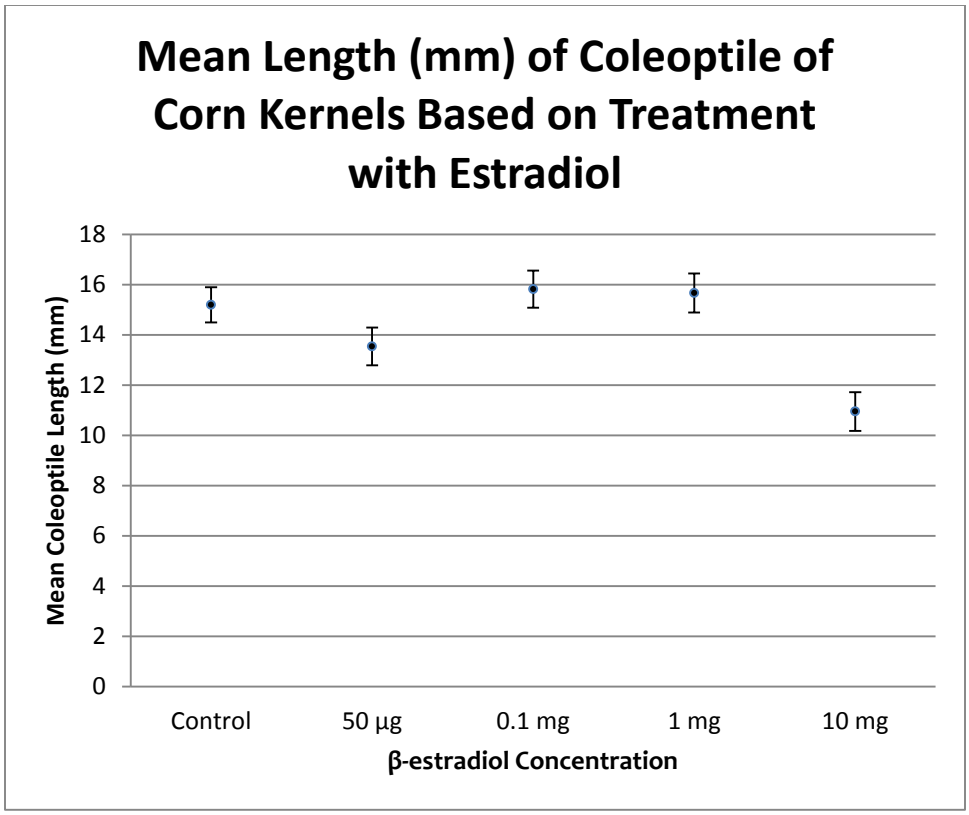

Figure 2.13. The mean length (mm) of the coleoptile of 5 day old corn (*Zea mays* L.) seedlings germinated and grown in different concentrations of β-estradiol. Vertical bars are based on a 97.5% confidence interval.

Most of the treatments contained kernels that had relatively similar numbers of adventitious roots as well as a similar appearance. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was a significant difference in the number of adventitious roots after five days of growth. A p-value of <.0001 was determined using a confidence interval of 97.5%. Using a Tukey's Studentized Range test for mean number of adventitious roots based on treatment and comparisons significant at alpha 0.025, it was determined that the 10 mg treatment was statistically different from every other treatment (50 µg, 0.1 mg, 1.0 mg, or control). It was also determined that the 0.1 mg treatment was significantly different from the control. The mean number of adventitious roots (Fig 2.14) was 4.95 with a standard error of 0.27 for the control, 5.23 with a standard error of 0.36 for the 50 µg treatment, 6.43 with a standard error of 0.40 for the 0.1 mg treatment, 5.29 with a standard error of 0.34 for the 1.0 mg treatment, and 3.40 with a standard error of 0.34 for the 10 mg treatment. The greatest mean number of adventitious roots was recorded in the 0.1 mg treatment group and the least mean number of adventitious roots was recorded in the 10 mg treatment group. The difference between the means was 3.03 adventitious roots.

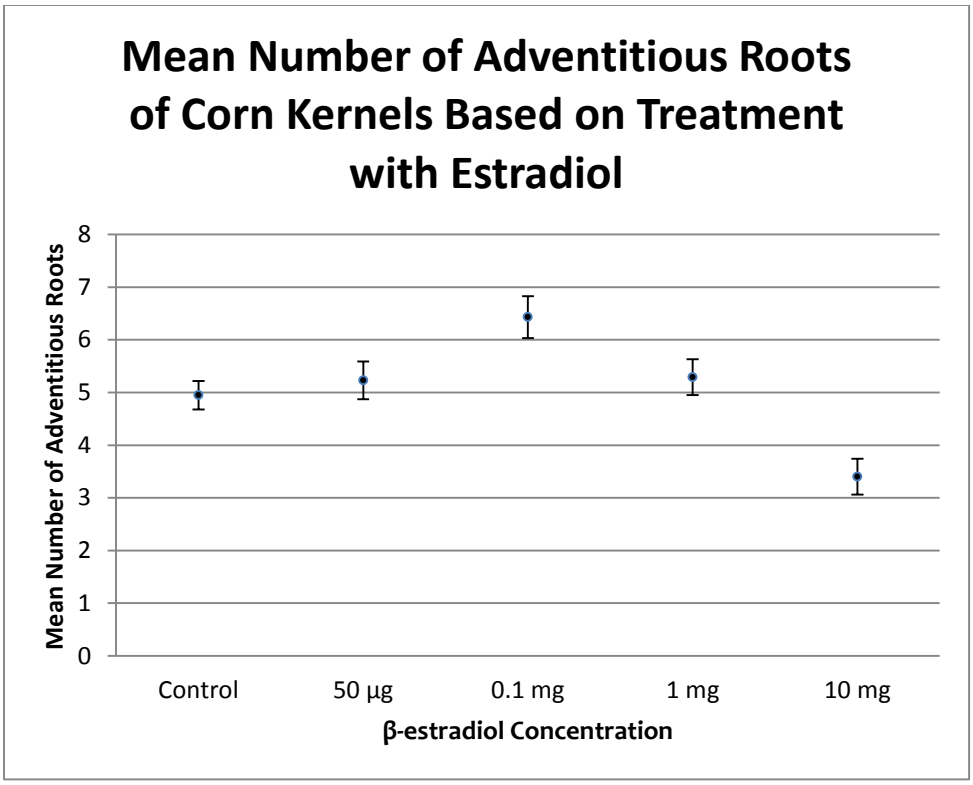

Figure 2.14. The mean number of adventitious roots of 5 day old corn (*Zea mays* L.) seedlings germinated and grown in different concentrations of β-estradiol. Vertical bars are based on a 97.5% confidence interval.

There were 500 kernels used to complete this experiment. It was observed that the 10 mg β-estradiol treatment group displayed a significant lack of germination in all three trials. The other treatment groups were relatively similar in both the hour of germination and the overall percentage. If a kernel germinated it was assigned a 1 and if a kernel did not germinate it was assigned a 0. The averages were then used to determine the overall percentage of germination. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was a significant difference in the overall percentage of germination after 96 hours based on the treatment the kernels were subjected to. A p-value of <.0001 was determined using a confidence interval of 97.5%, indicating that at least one of the treatments was significantly different than the others. Using a Tukey's Studentized Range test for overall percentage of germination based on treatment and comparisons significant at alpha 0.025, it was determined that the 10 mg treatment was statistically different from every other treatment (50 µg, 0.1 mg, 1.0 mg, or control). The overall percentage of germination (Figure 2.15) was 92% with a standard error of 0.03 for the control, 90% with a standard error of 0.03 for the 50 µg treatment, 97% with a standard error of 0.02 for the 0.1 mg treatment, 92% with a standard error of 0.03 for the 1.0 mg treatment, and 73% with a standard error of 0.04 for the 10 mg treatment. The greatest overall percentage of germination was recorded in the 0.1 mg treatment group and the least overall percentage of germination was recorded in the 10 mg treatment group. The difference between the means in overall germination was 24%.

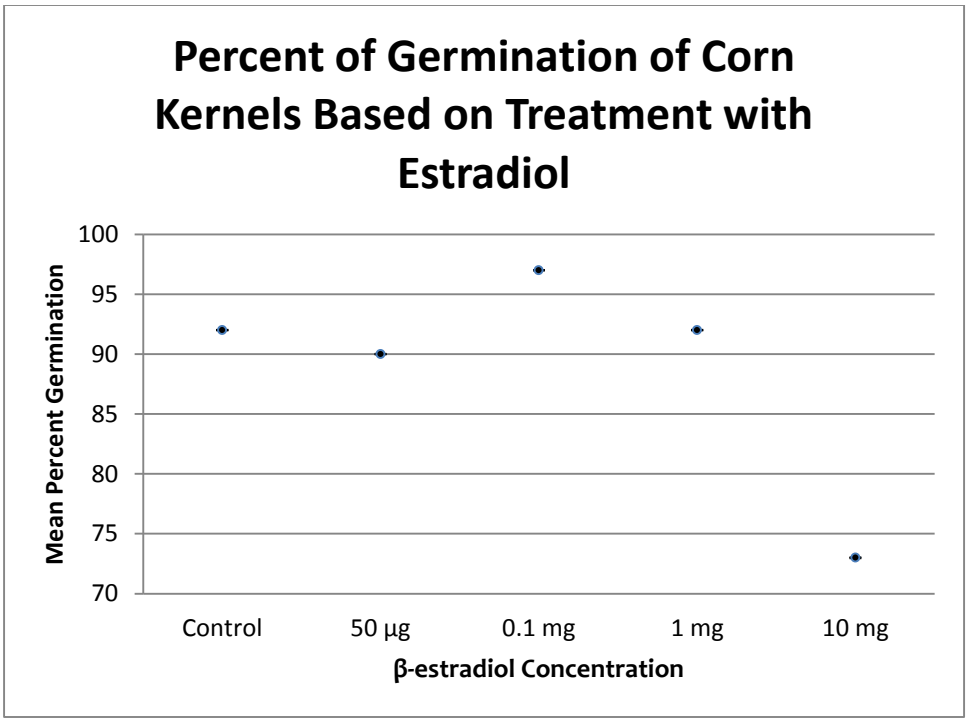

Figure 2.15. The overall percentage of germination after 96 hours in various concentrations of β-estradiol. A corn (*Zea mays* L.) kernel that germinated was assigned a 1 and a kernel that did not germinate was assigned a 0. Vertical bars are based on a 97.5% confidence interval.

Discussion

According to the results, β-estradiol has a significant effect on the germination of *Zea mays* L. kernels. At the highest concentration of the hormone, 10 mg/mL, the overall germination was reduced to 73%. This indicates that at some level β-estradiol is toxic to the kernels at high concentrations. At smaller concentrations it appears that β-estradiol causes a slight escalation in overall germination, 97% in 0.1 mg when compared to the control at 92%. Germination is a key phase in the initiation of plant growth. If germination is being inhibited by high concentrations of the hormone, this could indicate that kernels sown in fields that are exposed to contaminated water or soil, may have reduced germination rates and therefore reduced overall yield. It can also be established that high levels of β-estradiol appears to cause a significant delay in the germination process. This can be seen in the results above which shows the 10 mg treatment group taking on average 66 hours to germinate, while kernels that were exposed to the 0.1 mg treatment group took on average 48 hours to germinate compared to the control that averaged 54 hours.

These results seem to fall in line with the plant species *Lactuca sativa* L. (lettuce) and *Lycopersicon esculentum* Mill. (tomato) which also exhibited a reduction in the overall rate of germination when exposed to β-estradiol (D'Abrosca et al 2008). However, the results are at odds with the outcomes of experiments previously performed on both *Cicer arietinum* L. (chickpea) and *Daucus carota* L. (carrot) which both displayed an increase in the overall rates of germination (Erdal and Dumlupinar

2010). Both *L. sativa* and *L. esculentum* are dicots, which demonstrated the same results as *Z. mays* which is a monocot. Therefore it cannot be assumed that the reason that there was a difference was due to a difference in monocots versus dicots (D'Abrosca et al 2008). Legumes such as *C. arietinum* are known to have high levels of endogenous compounds known as phytoestrogens (Erdal and Dumlupinar 2010). These compounds are extremely close in chemical structure and nature to β-estradiol. The influence on germination may be positive due to that close connection and increased binding of phytoestrogen receptors with β-estradiol. The negative effect of β-estradiol on the corn kernels observed in this experiment would indicate that the hormone is inhibiting the mechanisms of germination either by binding to receptors or in some other way down-regulating the gene expression for cellular components involved in the process. Additionally, it has been also been suggested that these steroids might be involved in a disturbance of the Na/K balance of plant cells (Agarwal 1993).

It was also observed in the results that β-estradiol had a significant effect on the primary root development. The average length of the primary root after 5 days of growth after germination in the 10 mg treatment group was only 5.83 mm compared to 31.75 mm in the control group. This same phenomenon was observed in both *H. annuus* and *S. lycopersicum* (Janeczko and Skoczowski 2005). This may illustrate that exposure to high concentrations of β-estradiol could inhibit the functioning of the primary root apical meristem. The process of cell elongation within the root could also be affected, which is a primary means for increasing length (Taiz and Zieger 2006). Adventitious roots begin to grow after the primary, so perhaps by this point in time the β-estradiol

had denatured to a point where it was no longer causing inhibition of root growth. Several seedlings exposed to the highest concentration of β-estradiol from this portion of the experiment would exhibit regular coleoptile growth, but would have severely limited or no root growth. This indicates that the coleoptile growth is not hindered quite like the primary root. This could perhaps come from the fact that the primary root is the first to emerge followed by the coleoptile, about one day later (Nielson 2010). With the initial imbibition of water containing β-estradiol directed towards root development, the coleoptile may not be as directly affected. The results of this experiment also showed that there was a significant reduction in the coleoptile length as well as in the number of adventitious roots of the 10 mg treatment goup. While statistically significant, from a purely observational standpoint these did not appear as starkly contrasting when compared to the other treatment groups as the primary root measurements did.

It should also be noted that in the dishes containing the highest concentrations of β-estradiol there was occasional mold growth even though the procedures were carried out under as sterile conditions as possible. This may be indicative of a relationship between fungi and β-estradiol. Previous reports have shown that in some instances fungal growth can be stimulated by the hormone (Stoka 1999). The growth of mold was not observed in any other dishes.

Chapter 3: The effect of β-estradiol on growth of corn (*Zea mays* L.)

In mammals, β-estradiol plays a key role in controlling the processes revolving around development and reproduction as well as being involved in the control of both mineral and protein metabolism (Carreau et al. 2002). Knowing that this hormone is a contaminant in several key water sources for plants, the question arises whether or not there is also an effect upon plant growth and development (Islam and Tanaka 2004). Several studies have been carried out on different plant species in order to determine this.

In sunflower seedlings (*Helianthus annuus* L.), β-estradiol concentrations of 1 µg per plant increased overall shoot growth, but was shown to inhibit overall root growth (Janeczko and Skoczowski 2005). In tomato (*Lycopersicon esculentum* Mill.) seedlings the hormone was shown to reduce overall root growth as well as the overall number of roots present in nutrient solutions with a 1 µM concentration of β-estradiol (Janeczko and Skoczowski 2005). In alfalfa plants (*Medicago sativa* L.) the lower concentrations of β-estradiol used (0.005-0.5 $\mu g \cdot dm^{-3}$) in the experiment favored increased growth, while the higher concentrations used (50-500 $\mu g \cdot dm^{-3}$) inhibited growth of both roots and shoots (Janeczko and Skoczowski 2005). In chickpeas (*Cicer arietinum* L.), the hormone significantly enhanced the root and shoot growth of the seedlings at concentrations of 10^{-4}, 10^{-9}, 10^{-12}, and 10^{-15} M (Erdal and Dumlupinar 2010).

Thus, there appears to be a wide variation in the effects of β-estradiol on the growth of different plant species. This suggests that how a plant responds depends on

the species of plant and the concentration of β-estradiol that the plant is exposed to. The goal of this experiment was to determine if β-estradiol had an effect on the growth of corn (*Zea mays* L.) seedlings.

Materials and Methods

Fifty maize (*Z. mays*) kernels were surface sterilized with a 20% household bleach and then placed into petri dishes lined with filter paper. Fifteen mL of sterile water was added and the kernels were allowed to germinate in the dark at 26 ^{o}C for three days. Fifty individual growth tubes (Blowmolded cells D16 L 2"x7", Stuewe & Sons Inc.) were lined with triple ply cheese cloth. Tubes were then completely filled with vermiculite and randomly assigned to one of four concentrations of β-estradiol or a control. Tubes were then placed into a holding tray. One germinated kernel was placed into each of the 50 tubes of vermiculite and covered with additional vermiculite (Fig 3.1). Six mg of β-estradiol was dissolved in 1 mL of 95% ethanol and then diluted with sterile water to 10 mg/L, 1.0 mg/L, 0.1 mg/L and 50 µg/L. Each tube was treated with 50 mL of the steroid treatment or sterile water (control) and placed in an incubator at 26 ^{o}C. Incubator photoperiod was set at 12 hours of light followed by 12 hours of dark per day. After three days each tube received 20 mL of a nutrient nitrogen deficient solution (LaMotte Nutrient Solution 5940). The solution was made with 2 mL of 1 M magnesium sulfate, 10 mL of 0.05 M calcium phosphate, 5 mL of 0.5 M potassium sulfate, 200 mL of 0.01 M calcium sulfate, 1 mL of iron-EDTA, and 1 mL of trace elements filled to 1 L of sterile water. On days 4, 10, and 16 the seedlings received 20 mL of nutrient water. On days 7, 13, and 19 the seedlings received treatments of 50 mL of the assigned β-estradiol concentrations or sterile water for the control tubes (Fig 3.2). After 20 days the tubes were removed from the incubator. Total shoot system height (mm) and total root system length (mm) was measured (Fig 3.3) and

Figure 3.1. Fifty individual growth tubes (Blowmolded cells D16 L 2”x7”, Stuewe & Sons Inc.) were lined with triple ply cheese cloth, completely filled with vermiculite and randomly assigned to one of four concentrations of β-estradiol or a control. Tubes were then placed into a holding tray. One germinated kernel was placed into each of the 50 tubes and covered with additional vermiculite.

Figure 3.2. Corn (*Zea mays* L.) kernels were placed into a Thermo Scientific incubator (model 818) at 26^{o} C with a photoperiod set at 12 hours for 20 days. On days 4, 10, and 16 the seedlings received 20 mL of nutrient water. On days 7, 13, and 19 the seedlings received treatments of 50 mL of the assigned β-estradiol concentrations or sterile water for the control tubes.

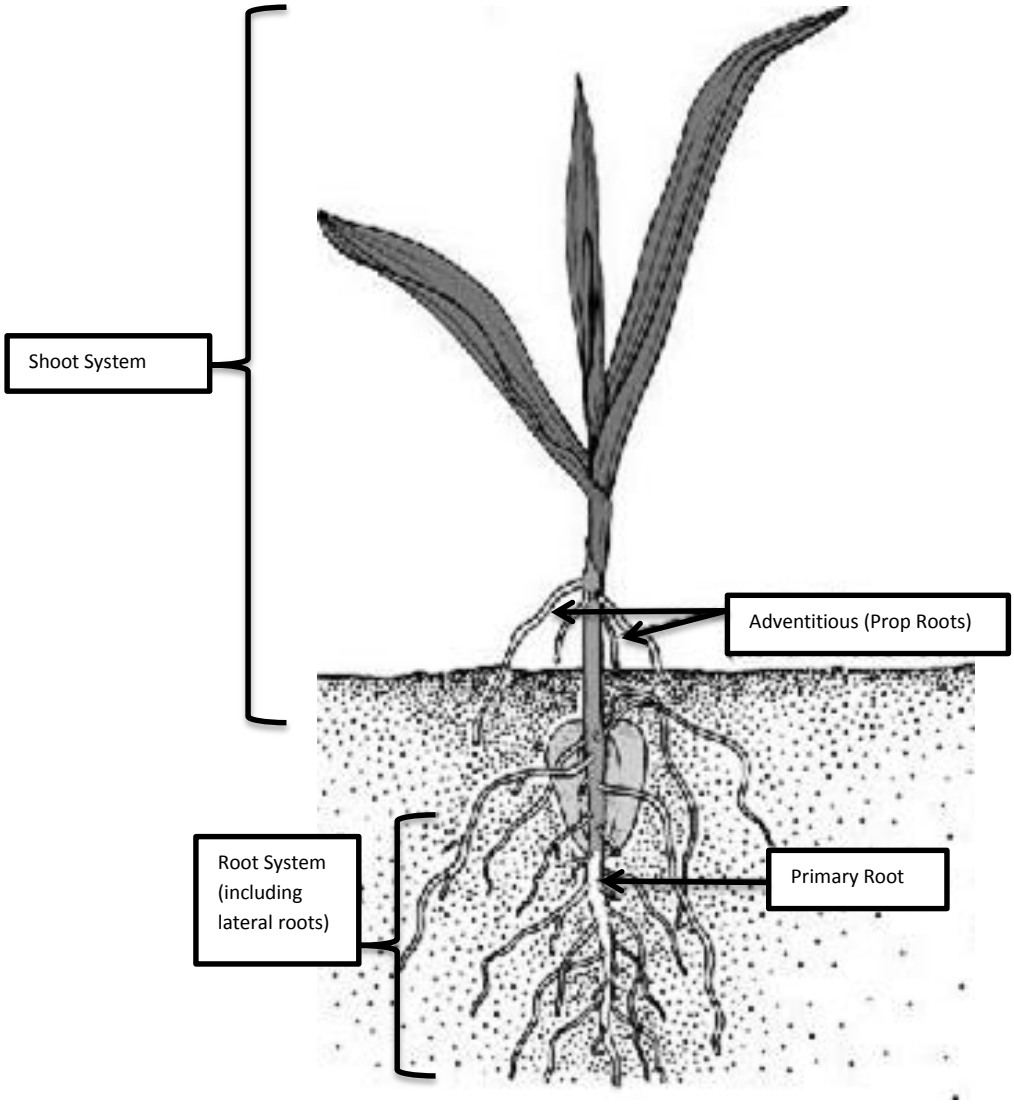

Figure 3.3. The corn (*Zea mays* L.) kernels were removed from the incubator after 20 days of growth in various concentrations of β-estradiol. Measurements (mm) were taken of both the length of the entire shoot system as well as the entire root system.

recorded. The total number of leaves was also recorded for each plant. The entire shoot system of each plant was removed with a scalpel and placed into a container with all other shoot systems from the same β-estradiol concentration and stored at -80 ^{o}C. Similar procedures were completed for roots. This entire procedure was repeated three times for each concentration, resulting in 150 plants.

Results

There were 150 plants used to complete this experiment. It was observed that most of the treatment groups were similar in overall appearance (Fig 3.4). The 10 mg treatment group did have some anomalies. One of the plants in this group had two sets of root systems, and no shoot system. There were others in the 10 mg group that had started to grow, but had died relatively early. These plants did not develop past the second emerging leaf and exhibited minimum shoot growth. Still others exhibited shallow root systems (Fig. 3.4) compared to all other treatments as well as poorly developed shoot systems. Many of the leaves of the shoot systems had begun to wither and die (Fig. 3.4).

Using SAS programming for one-way ANOVA statistical analysis it was determined that there was a significant difference in the mean height of the shoot system after 20 days of growth in a specific concentration of β-estradiol (or sterile water). A confidence interval of 97.5% a p-value of 0.0077 was determined. Using a Tukey's Studentized Range test for mean height of the shoot system, based on treatment and comparisons significant at alpha 0.025, it was determined that the 10 mg treatment was statistically different from the control and 1.0 mg treatment groups. The 10 mg treatment group was not significantly different from either the 0.1 mg or 50 µg treatment groups. The mean height (Fig 3.5) of the shoot system for the control was 393.78 mm with a standard error of 16.68, 368.88 mm with a standard error of 23.87 for the 50 µg treatment, 345.85 mm with a standard error of 27.76 for the 0.1 mg

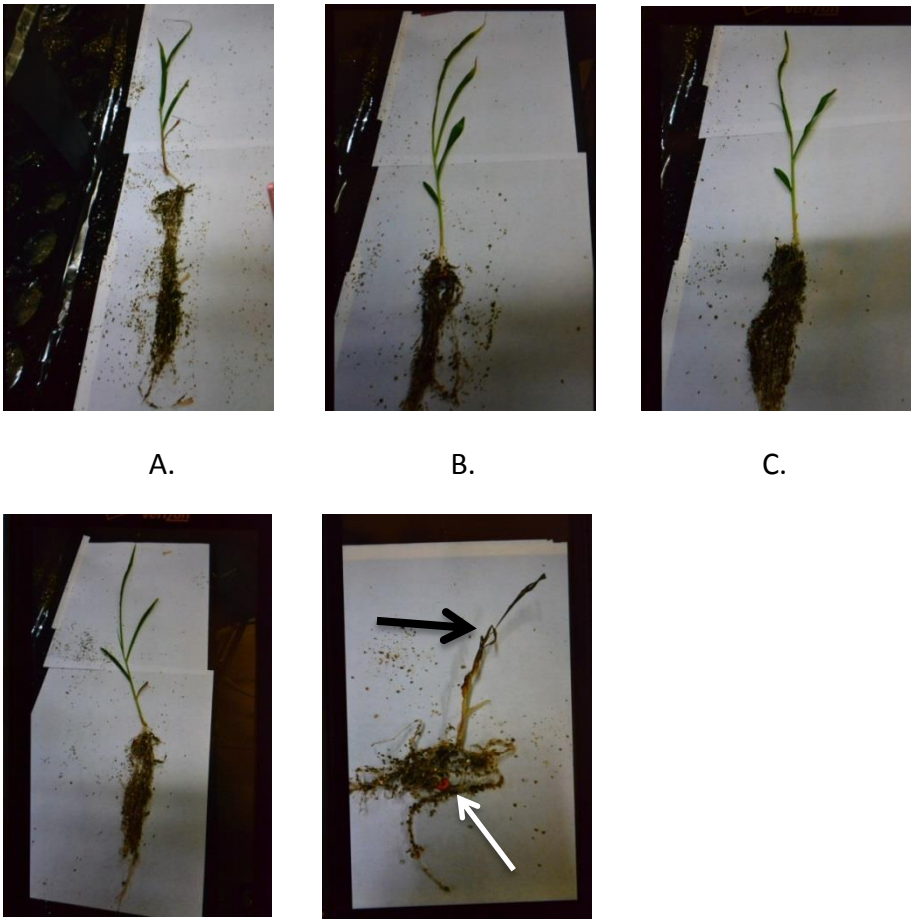

Figure 3.4. Corn (*Zea mays* L.) plants after 20 days of growth in 50 µg/mL, 0.1 mg/mL, 1.0 mg/mL and 10 mg/mL concentrations of β-estradiol (B,C,D,E respectively) or in a control solution (A). Note the shallow root system (white arrow) that was typical for the 10 mg/mL treatment group and that the leaves on the shoot system (black arrow) were starting to die (E).

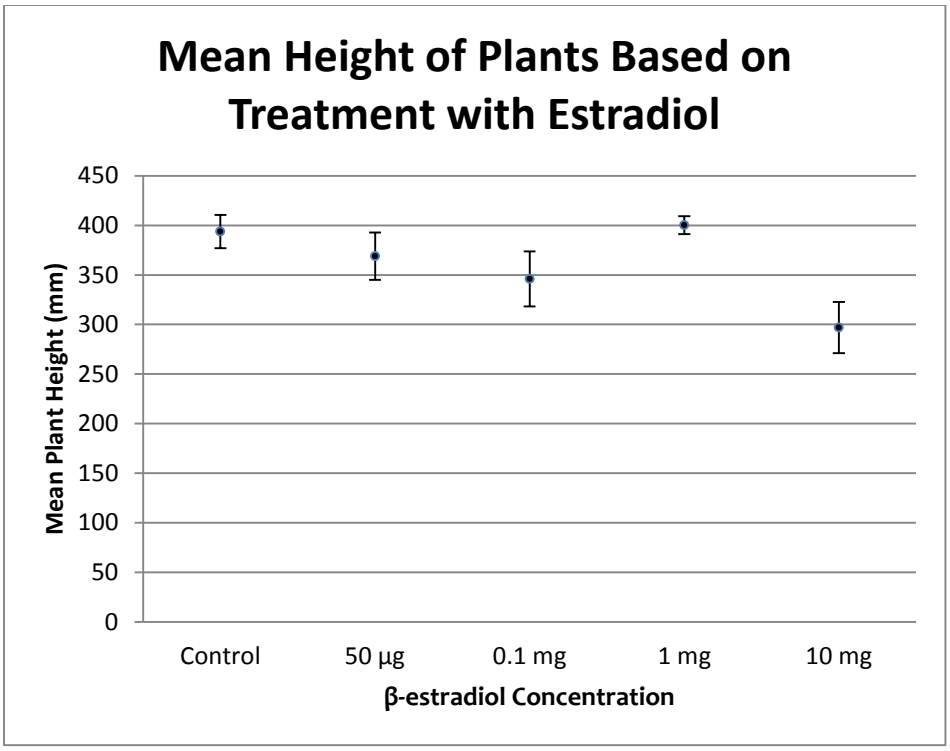

Figure 3.5. The mean shoot system height of corn (*Zea mays* L.) seedlings after 20 days of growth in various concentrations of β-estradiol. There are 30 plants in each treatment group for a total of 150 plants. The vertical bars are based on a 97.5% confidence interval.

treatment, 400.2 mm with a standard error of 9.18 for the 1.0 mg treatment, and 296.9 mm with a standard error of 25.83 for the 10 mg treatment. The greatest mean height (mm) was recorded in the 1.0 mg treatment group and the least mean height (mm) was recorded in the 10 mg treatment group. The greatest difference between the means in plant height was 103.3 mm.

It was observed that the majority of plants had very similar root systems both in overall structure and size. The only group that stood out visually from the rest and exhibited a few irregularities was the 10 mg β-estradiol treatment group (Fig 3.4). The roots of the 10 mg group were usually shorter in overall length and overall girth when compared to the rest of the treatment groups. There was one seedling in the 10 mg group that actually had two root systems and no shoot system. There were roots coming out of both ends of the kernel. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was a significant difference in the mean length of the entire root system after 20 days of growth in assigned β-estradiol concentrations. Using a confidence interval of 97.5% a p-value of 0.0174 was determined. Using a Tukey's Studentized Range test for mean length of the entire root system based on treatment and comparisons significant at alpha 0.025, it was determined that the 10 mg treatment was statistically different from the 0.1 mg treatment group. The 10 mg treatment group was not significantly different from any other treatment groups. The 0.1 mg group was not significantly different from any other treatment group. The mean length (Fig 3.6) of the entire root system (mm) was 151.7 mm with a standard error of 3.94 for the control, 156.7 mm with a standard error of

3.75 for the 50 µg treatment, 176.8 mm with a standard error of 10.13 for the 0.1 mg treatment, 158.6 mm with a standard error of 4.03 for the 1.0 mg treatment, and 149.4 mm with a standard error of 6.03 for the 10 mg treatment. The longest mean root system was recorded in the 0.1 mg treatment group and the shortest mean root system was recorded in the 10 mg treatment group. The greatest difference between the means in overall root system length was 27.4 mm.

It was observed that there was not a noticeable difference in the number of leaves on the plants between the control and 1.0 mg, 0.1 mg, and 50 µg treatment groups (Fig 3.4). The 10 mg treatment group did exhibit some plants with wilting leaves and dying shoot systems. Plants in treatment groups other than the 10 mg appeared to have relatively similar numbers of leaves on their shoot systems. The leaves appeared to have the same appearance, size, and coloration as well. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was not a significant difference in the mean number of leaves of plants after germination followed by 20 days of growth based on the treatment the kernels were subjected to. Using a confidence interval of 97.5% a p-value of 0.2771 was determined, indicating that none of the treatments were significantly different from the others. The mean number of leaves (Fig. 3.7) was 3.9 with a standard error of 0.316 for the control, 4 with a standard error of 0.000 for the 50 µg treatment, 3.9 with a standard error of 0.385 for the 0.1 mg treatment, 4.4 with a standard error of 0.489 for the 1.0 mg treatment, and 3.5 with a standard error of 0.500 for the 10 mg treatment. The greatest mean number of leaves was recorded in the 1.0 mg treatment group and the least mean number of leaves was

recorded in the 10 mg treatment group. The greatest difference between the means was 0.9 leaves.

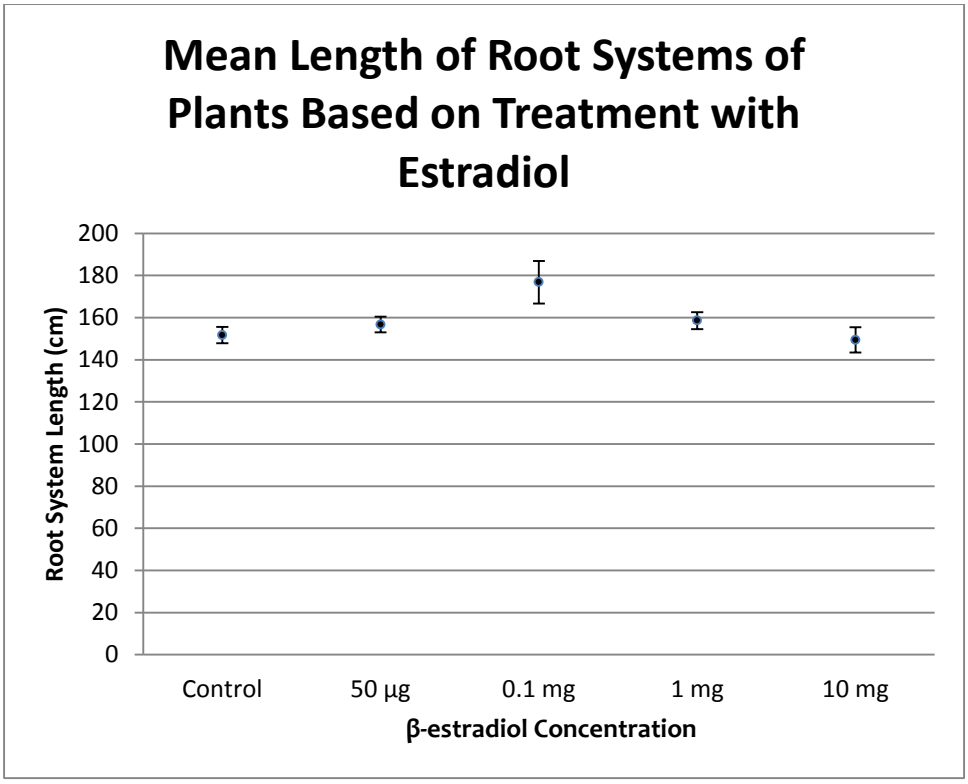

Figure 3.6. The mean length of corn (*Zea mays* L.) root systems of plants after 20 days of growth in various concentrations of β-estradiol. Thirty plants were in each treatment group. The vertical bars are based on a 97.5% confidence interval.

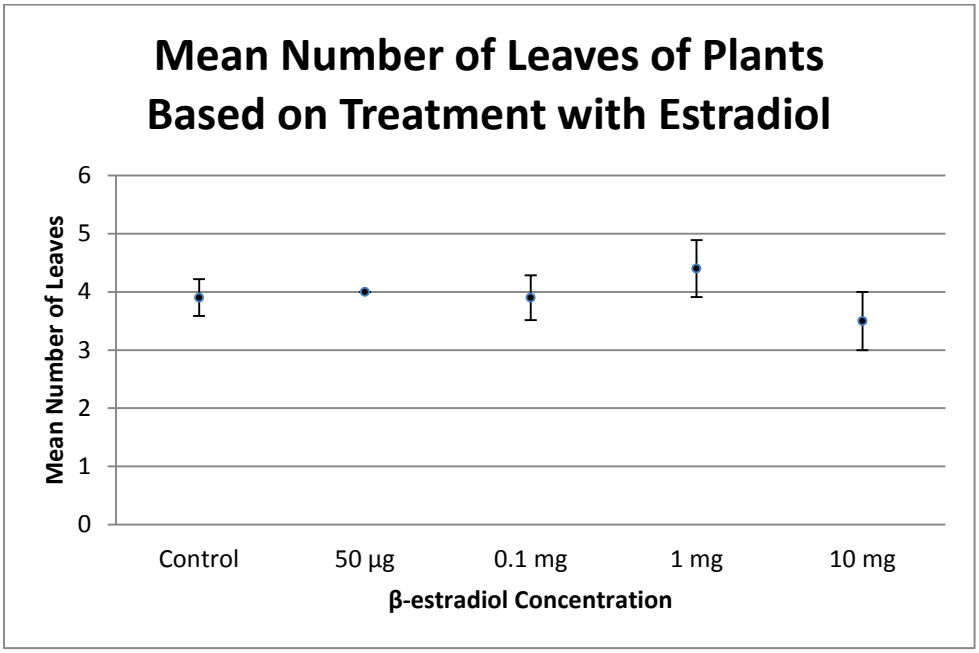

Fig 3.7. The mean number of corn (*Zea mays* L.) leaves of plants after 20 days of growth in various concentrations of β-estradiol. Thirty plants were in each treatment group. The vertical bars are based on a 97.5% confidence interval.

Discussion

According to the results there was an effect on both the height of the shoot system (mm) and the length of the root system (mm) of *Zea mays* L. due to exposure to β-estradiol. The highest β-estradiol concentration used caused a significant decrease in both the overall shoot height as well as the overall root system length. The 10 mg β-estradiol treatment group had a mean overall shoot length of 296.9 mm which was significantly lower than the control group at 393.78 mm. The 10 mg treatment group had a mean overall root system length of 149.4 mm which was lower than all of the other groups, and significantly lower than the 0.1 mg treatment group at 176.8 mm. This appears to follow the same results that were found in studies in some other species of plants. For example, in tomato seedlings (*L. esculentum*) β-estradiol concentrations of 1 μM was shown to reduce overall root growth as well as the overall number of roots present (Janeczko and Skoczowski 2005). In alfalfa (*M. sativa*) plants, the lower concentrations of β-estradiol used (0.005-0.5 $\mu g \cdot dm^{-3}$) in the experiment favored increased growth, while the higher concentrations used (50-500 $\mu g \cdot dm^{-3}$) inhibited growth of both roots and shoots (Janeczko and Skoczowski 2005).

The negative effect of β-estradiol on the corn plants observed in this experiment was predominantly found within the 10 mg/mL concentration group and would indicate that the hormone (at high concentrations) may be inhibiting the mechanisms of cell elongation or cellular division either by binding to receptors or in some other way down-regulating the gene expression for cellular components involved in the processes.

Previous studies have shown that phenolic compounds (such as β-estradiol) are well established regulators of gene expression (Shore et. al 1992). The exact mechanism(s) by which expression is regulated by β-estradiol is still under investigation. There are over 8,000 known phenolic compounds in plants and their functions range from cell wall structure, plant defense, color of woods and barks, flower color, and flavors within plant tissues. Examples of some phenolic compounds commonly found in vascular plants include flavonols, anthocyanins, tannins, lignins, and salicylic acid.

While the highest concentration of β-estradiol used (10 mg/mL) consistently had negative impacts on various aspects of *Zea mays* L. seedling growth, the lower concentrations (1.0 mg/mL, 0.1 mg/mL, and 50 μg/mL) were not statistically significant from the control group. In fact, the longest mean root system length was recorded in the 0.1 mg/mL treatment group, while the greatest mean shoot system height was recorded in the 1.0 mg/mL treatment group. Although, there have been relatively few studies conducted on the growth of plants in the presence of β-estradiol, it should be noted that the plants used in previous studies were dicotyledonous species (*H. annuus*, *L. esculentum*, *M. sativa*, *C. arietinum*). *Zea mays* L. is a monocotyledonous species. There may be a difference in the physiology and pathways determining the growth of tissues between dicotyledonous and monocotyledonous species, although this has not yet been investigated.

It should also be noted that in the blow-molded cells containing the highest concentrations (10 mg) of β-estradiol there was occasional mold growth even though

the procedures were carried out under as sterile conditions as possible. Three tubes in this treatment group exhibited the mold growth. This is indicative of a relationship between fungi and β-estradiol. Previous reports have shown that in some instances fungal growth can be stimulated by the hormone (Stoka 1999). The growth of mold was observed in one blow-molded cell in the 1.0 mg concentration as well, however, it was never found in any of the other treatment groups.

Chapter 4: The effect of β-estradiol on chlorophyll concentration of corn (*Zea mays* L.)

The presence of chlorophyll in the chloroplasts of plants is what allows them to be autotrophic , in that they are able to absorb light energy from the sun and convert it to chemical energy (Campbell et al. 2011). The amount of chlorophyll present in plant tissues can be a good indicator of how well the plant will be able to photosynthesize and produce sugars as well as other carbohydrates, and therefore indicates how well a plant may be able to grow. An increase or decrease in the amount of chlorophyll present would have a direct influence on the amount of resources available for a plant to use for growth. Producing new tissues will allow the plant to increase the height of the shoot system, length of the root system, and eventually lead to flowering and seed production (D'Abrosca et al. 2008).

Previous studies have been conducted to determine whether or not β-estradiol had an effect on the amount of chlorophyll present in the tissues of various species of plants. In *Daucus carota* L. the presence of β-estradiol at concentrations of 3-12 $mg \cdot dm^{-3}$ was shown to favor chlorophyll synthesis in leaves (Janeczko and Skoczowski 2005). The same phenomenon of an increased production of chlorophyll was observed using β-estradiol concentrations of 10^{-12} to 10^{-7} M in the algal cells of *Chlorella vulgaris* M. Beijerinck (a type of green alga) that resulted in increased sugar and protein content in these cells as well (Janeczko and Skoczowski 2005). In another study the presence of β-estradiol at concentrations of 1 µM and 1 nM resulted in a 30-50% reduction of chlorophylls and carotenoids in *Lactuca sativa* L. leaves which then led to a 50%

reduction in the sugar content (D'Abrosca et al. 2008). The results of these studies indicate that the effects of β-estradiol on the amount of chlorophyll in plants and other photosynthetic organisms depends on the specific species as well as the β-estradiol concentration. The goal of this experiment was to determine what effect different concentrations of β-estradiol would have on the amount of chlorophyll produced in *Zea mays* L. leaves.

Materials and Methods

Samples for this experiment were taken from the leaves of *Zea mays* L. that had been collected and frozen at -80 C^{o} after the experiment on the effects of β-estradiol on growth (Chapter 3). The shoot systems were thawed and leaves from each individual plant were collected until a 0.1 g sample was obtained. Chlorophyll extraction was carried out according to the process outlined in Investigating Plant Physiology by Camellia Okpudu (2001). Each 0.1 g tissue sample was placed into an individual 1.5 ml Eppendorf tube along with 1 mL of anhydrous methanol (Sigma-Aldrich Co., St. Louis, MO, USA). Once filled with anhydrous methanol, tubes were vortexed, allowed to sit for 30 minutes, and then vortexed again. Samples were then centrifuged at 10,000G for 10 minutes. Half of a mL of supernatant from each tube was collected and added to a new Eppendorf tube and the volume was brought to 1 mL with distilled water. A spectrophotometer (Biorad Smartspec Plus) was used to read the absorbance of diluted samples at three separate wavelengths: 420 nm, 645 nm, and 663 nm. The absorbance at 420 nm was used to determine the optimum dilution of samples. Samples that are too concentrated would not provide accurate readings from the spectrophotometer. Mass of chlorophyll/mL for each sample tube was calculated using the following equation from Okpodu (2001):

micrograms chlorophyll/mL$= (20.2 \times A_{645}) + (8.02 \times A_{663})$,

where A_{645} is the optical density measured from absorbance at 645 nm, and A_{663} is the optical density measured from absorbance at 663 nm

.

Dilution factors were then determined by taking total sample tube volumes and dividing these by the volume of original extract used. Total micrograms of chlorophyll were determined by taking the dilution factor and multiplying it by the mass of chlorophyll/mL. There was a 0.1 g sample taken from each plant used during the growth experiment, leading to a total of 150 samples.

Results

There were 150 plant samples used to complete this experiment. It was observed that there was quite a bit of variation in the depth of color of the samples used in the spectrophotometer. There was variation in the visual appearance not only between the treatment groups, but within them as well. Overall the darkest (most saturated appearing) samples seemed to come from the 10 mg samples. Using SAS programming for one-way ANOVA statistical analysis it was determined that there was not a significant difference in the mean amount of chlorophyll in µg/mL after 20 days of growth in assigned β-estradiol concentrations. Using a confidence interval of 97.5% a p-value of 0.5381 was determined, indicating that none of the treatments were significantly different from the others. The mean amount of chlorophyll in µg/mL (Figure 4.1) was 5.72 µg with a standard error of 0.895 for the control, 5.60 µg with a standard error of 0.560 for the 50 µg treatment, 6.81 µg with a standard error of 1.396 for the 0.1 mg treatment, 6.18 µg with a standard error of 0.828 for the 1.0 mg treatment, and 7.63 µg with a standard error of 0.934 for the 10 mg treatment. The greatest mean amount of chlorophyll in µg/mL was recorded in the 10 mg treatment group and the least mean amount of chlorophyll in µg/mL was recorded in the 50 µg treatment group. The greatest difference between the mean amount of chlorophyll in µg/mL was 2.03 µg.

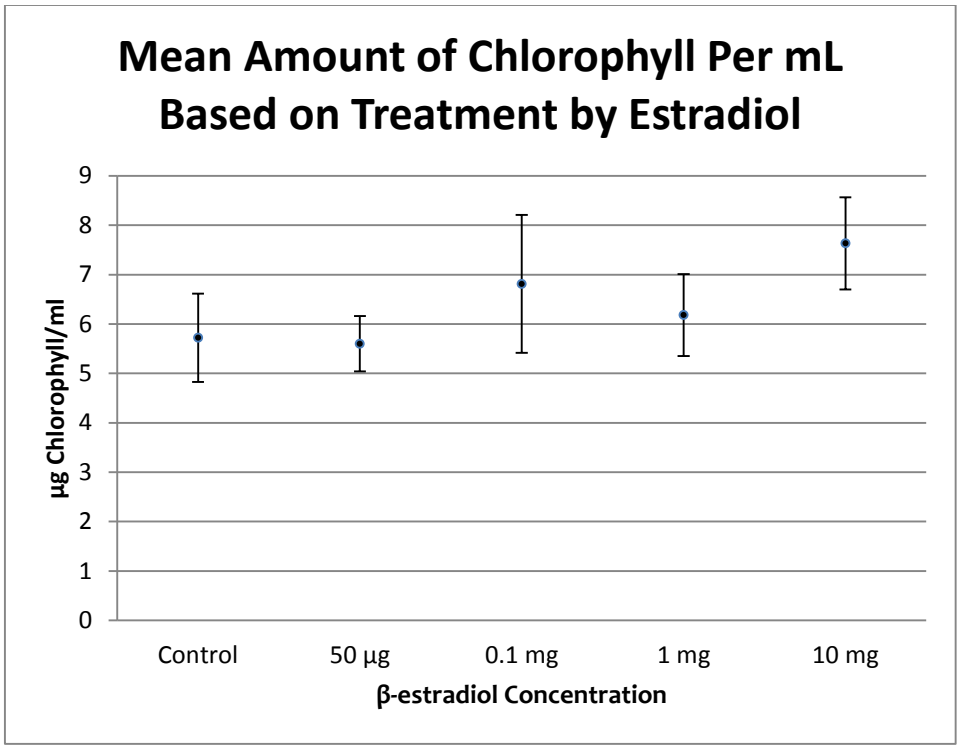

Figure 4.1. The mean amount of chlorophyll in leaves from corn seedlings (*Zea mays* L.) after 20 days of growth in various concentrations of β-estradiol. There were 30 plants per treatment. The vertical bars are based on a 97.5% confidence interval.

Discussion

According to the results there was not a significant effect of β-estradiol on the amount of chlorophyll in the leaves of corn plants grown in various concentrations of the hormone for 20 days. There was a wide variation in the amount of chlorophyll amongst the samples within each concentration group. The variations were found in all of the treatment levels as well as the control group. The high level of variation in chlorophyll levels within each of the treatment groups could indicate that there is no correlation between the β-estradiol and chlorophyll production in *Zea mays* L. It could also be indicative of experimental error and follow up studies could be conducted to corroborate the results. It should be noted that the 10 mg group did have the highest mean amount of chlorophyll. Even though the chlorophyll levels exhibited in the 10 mg group were not significant from the other concentrations, the increase in chlorophyll content is consistent with the experiments carried out on *D. carota* and *C. vulgaris* (Janeczko and Skoczowski 2005). These species also exhibited an increase in the amount of chlorophyll with the presence of the hormone.

Chapter 5: Conclusion

Recap water pollution and effects of β-estradiol

Ecosystems consist of complex and intricate relationships between organisms and their biotic and abiotic surroundings. When there is a change to any part of the biotic or abiotic resources in an ecosystem, it can be drastically altered (Islam and Tanaka 2004). One of the most important resources for an ecosystem is water. As a key reactant in photosynthesis, water helps to form the base of any ecosystem by providing the necessary resources for photosynthetic organisms, which then in turn provide sustenance for heterotrophic organisms as well as oxygen for aerobic organisms (Campbell et al. 2011).

The pollution of water sources and the damaging results have been well documented over the past few years. To date, the greatest amount of waste that ends up in aquatic ecosystems is sewage (Islam and Tanaka 2004). Sewage effluent can include waste from industry, municipalities, animal remains, slaughterhouses, domestic baths and kitchens, fecal matter and effluent from numerous other sources (Islam and Tanaka 2004). Included among the sewage categories is a class of compounds known as Reproductive Endocrine Disruptors, or REDs. Several studies have been conducted that show where there are high levels of REDs in habitats there tends to be disruptions in the morphology and physiology of the organisms that reside there (Islam and Tanaka 2004). Examples of synthetic and naturally occurring chemicals that act as REDs include estrogenic and anti-androgenic substances. There are also chemicals that are not

hormonal in nature but can have estrogenic properties, such as alkyphenols, industrial pesticides, and chlorinated hydrocarbons (Islam and Tanaka 2004). One of the most common estrogenic sources in waste water effluent comes from the urine of women who take birth control pills. These hormone treatments contain the synthetic ethynylestradiol as well as significant amounts of 17β-estradiol (Batty and Lim 1999).

Recap of previous experiments, and their results.

In mammals, β-estradiol plays a key role in controlling the processes revolving around development and reproduction as well as being involved in the control of both mineral and protein metabolism (Carreau et al. 2002). Knowing that this hormone is a contaminant in several key water sources for plants, the question arises whether or not there is also an effect upon plant growth and development (Islam and Tanaka 2004). Several studies have been carried out on different plant species in order to determine this.

Previous investigations have examined the effect that β-estradiol has on seed germination in a wide variety of different plant species. β-estradiol decreased the germination percentage of lettuce, carrot, and tomato seeds (*Lactuca sativa* L., *Daucus carota* L., and *Lycopersicon esculentum* Mill. respectively), while in chickpea seeds (*Cicer arietinum* L.) the germination percentage increased. In sunflower seedlings (*Helianthus annuus* L.), β-estradiol concentrations of 1 µg per plant increased overall shoot growth, but was shown to inhibit overall root growth (Janeczko and Skoczowski 2005). In tomato

(*Lycopersicon esculentum* Mill.) seedlings the hormone was shown to reduce overall root growth as well as the overall number of roots present in nutrient solutions containing β-estradiol (Janeczko and Skoczowski 2005). In alfalfa plants (*Medicago sativa* L.), lower concentrations of β-estradiol favored increased root and shoot growth, while the higher concentrations used inhibited growth (Janeczko and Skoczowski 2005). In chickpeas (*C. arietinum*), the hormone significantly enhanced the root and shoot growth of the seedlings (Erdal and Dumlupinar 2010). Previous studies have also been conducted to determine whether or not β-estradiol had an effect on the amount of chlorophyll present in the tissues of various species of plants. In *D. carota* the presence of β-estradiol was shown to favor chlorophyll synthesis in leaves (Janeczko and Skoczowski 2005). The same phenomenon of an increased production of chlorophyll was observed in algal cells of *Chlorella vulgaris* M. Beijerinck (a type of green alga) (Janeczko and Skoczowski 2005). In another study the presence of β-estradiol exposure resulted in a 30-50% reduction of chlorophylls and carotenoids in *L. sativa* leaves (D'Abrosca et al. 2008).

Thus, there appears to be a wide variation in the effects of β-estradiol on the germination, growth, and chlorophyll content of different plant species. This suggests that the effect on the growth of plants will depend on the species of plant and the concentration of β-estradiol that the plant is exposed to. Although, there have been relatively few studies conducted on the growth and chlorophyll content of plants in the presence of β-estradiol, it should be noted that the plants used in previous studies were dicotyledonous species (*H. annuus*, *L. esculentum*, *M. sativa*, *C. arietinum*). *Zea mays* L.

is a monocotyledonous species. There may be a difference in the physiological and biochemical response to β-estradiol between dicotyledonous and monocotyledonous species, although this has not yet been investigated.

Recap of current germination experiment on corn (*Zea mays* L.).

Previously, no one had looked at the effect of β-estradiol on the germination, growth, and chlorophyll content of corn (*Zea mays* L.) and there were no publications prior to the start of this research in 2009. The goal of this experiment was to determine if β-estradiol had an effect on the germination of corn kernels and the growth of corn seedlings. Parameters investigated during germination were primary root length, coleoptile length, the number of adventitious roots, and the overall percentage of germination. The parameters examined during corn seedling growth were overall shoot system height, root system length, the number of leaves, and chlorophyll content.

For the experiment on the germination of *Z. mays* kernels in various concentrations of β-estradiol, it was determined that the 10 mg β-estradiol treatment group consistently exhibited a delay in germinating. For most other concentrations 48 hours was the optimal germination time, yet for the 10 mg group it was 72 hours. The 10 mg treatment was statistically significant from all other treatments (Fig 2.9). The 0.1 mg treatment group, which exhibited the fastest germination time, also tested significantly different from both the control and the 50 µg treatment.

It was observed that the kernels in the 10 mg treatment group were almost all lacking primary root growth (Fig 2.11). Most of the kernels had coleoptile growth and adventitious roots present, but the primary roots were highly undeveloped. The 10 mg group was the only one to exhibit these properties. It was determined that the 10 mg treatment was statistically different from all other treatments. Primary root length of the other treatments (50 μg, 0.1 mg, 1.0 mg, or control) were not significantly different from each other (Fig 2.12). The longest mean primary root length was recorded in the control treatment group while the shortest mean primary root length was recorded in the 10 mg treatment group.

It appeared that most of the germinated kernels across the different concentrations of β-estradiol had coleoptiles that were relatively similar in both length and girth. It was determined that the coleoptile length in the 10 mg treatment was statistically different from every other treatment except for the 50 μg treatment. None of the other treatments (0.1 mg, 1.0 mg, or control) were significantly different from each other as well as the 50 μg did not test significant against the other treatments. The longest mean coleoptile length was recorded in the 0.1 mg treatment group and the shortest mean coleoptile length was recorded in the 10 mg treatment group.

Most of the treatments contained kernels that had relatively similar numbers of adventitious roots as well as a similar appearance. The difference between the means of the 10 mg and 0.1 mg treatments was 3.03 adventitious roots. The 10 mg group had the least mean number of adventitious roots and was statistically different from every other

treatment (50 µg, 0.1 mg, 1.0 mg, or control). It was also determined that the 0.1 mg treatment was significantly different from the control in having the greatest mean number of adventitious.

Recap of current growth experiment on corn (Zea mays L.).

For the growth portion of the experiments, it was observed that most of the treatment groups were similar in overall appearance in reference to their shoot systems (Fig 3.4). The 10 mg treatment group did have some anomalies. A few started to develop a shoot system, but died relatively early. These plants did not develop past the second emerging leaf and exhibited minimum shoot growth. Still others exhibited poorly developed shoot systems. Many of the leaves of the shoot systems had begun to wither and die (Fig. 3.4). It was determined that the shoot length of 10 mg treatment was statistically different from the control and 1.0 mg treatment groups. The 10 mg treatment group was not significantly different from either the 0.1 mg or 50 µg treatment groups. The greatest mean shoot height (mm) was recorded in the 1.0 mg treatment group and the least mean height (mm) was recorded in the 10 mg treatment group.

It was observed that the majority of plants had very similar root systems both in overall structure and size. The only group that stood out visually from the rest and exhibited a few irregularities was the 10 mg β-estradiol treatment group (Fig 3.4). The roots of the 10 mg group were usually shorter in overall length and overall girth when compared to the rest of the treatment groups. There was one seedling in the 10 mg

group that actually had two root systems and no shoot system. This particular seedling had roots coming out of both ends of the kernel. It was determined that the 10 mg treatment was statistically different from the 0.1 mg treatment group. The 10 mg treatment group was not significantly different from any other treatment groups. The 0.1 mg group was not significantly different from any other treatment group.

It was observed that there was not a noticeable difference in the number of leaves on the plants (around 4 leaves) between the control and 1.0 mg, 0.1 mg, and 50 µ treatment groups (Fig 3.4). The 10 mg treatment group did exhibit some plants with wilting leaves and dying shoot systems. Plants in treatment groups other than the 10 mg appeared to have relatively similar numbers of leaves on their shoot systems. The leaves exhibited the same appearance, size, and coloration as well. It was determined that none of the treatments were significantly different from the others.

When visually observing the chlorophyll extract of *Z. mays* there was quite a bit of variation in the depth of color of the samples used in the spectrophotometer to determine chlorophyll content. There was variation not only between the treatment groups, but within them as well. Overall the darkest (most saturated appearing) samples seemed to come from the 10 mg samples. It was determined that none of the treatments were statistically significantly different from the others.

Summary of germination and growth experiments on corn (*Zea mays*)

Overall, the experimental results seem to indicate that higher concentrations of β-estradiol (10 mg/L) had a negative impact on aspects of both the germination and

seedling growth of *Z. mays.* The germination of kernels was significantly altered by the highest dose of β-estradiol. The mean number of hours before germination occurred for the 10 mg treatment group was almost a full day later than the lower concentrations. The overall percentage of germination was also drastically lower than the other treatment groups. The 10 mg treatment group averaged 72 percent and all the others averaged between 90 and 100 percent germination. This may be indicative of the hormone inhibiting the mechanisms of germination either by binding to receptors or in some other way down-regulating the gene expression for cellular components involved in the process. Another consistent abnormality observed in the 10 mg treatment group was either underdeveloped growth or the absence of primary root growth. This may illustrate that exposure to high concentrations of β-estradiol could inhibit the functioning of the primary root apical meristem. The process of cell elongation within the root could also be affected, which is a primary means for increasing length (Taiz and Zieger 2006).

The same trends of stunted root development were observed in the growth of *Z. mays* seedlings exposed to the highest concentration of β-estradiol. Statistically, the 10 mg treatment group had a much smaller overall root system length than the other treatment groups. This again suggests the hormone may be affecting some aspect of the root apical meristem and cell elongation through inhibition of the genes, or other aspects of cell regulation. Surprisingly, the hormone β-estradiol did not have a statistically significant effect on either the number of leaves or the chlorophyll content of the seedlings. With the significant alterations in the root systems, it was expected

that portions of the shoot system would also be affected. While, the total shoot system height was reduced in the 10mg treatment group, the other variables measured (number of leaves and chlorophyll content) did not test statistically significant from the other treatment groups.

It should be noted that while the 10 mg treatment group showed consistent inhibition among most of the parameters looked at in both the corn kernel germination and corn seedling growth experiments, the 0.1 mg treatment group almost always exhibited the highest measurements in all of the parameters observed including: mean hour of germination, coleoptile length, number of adventitious roots, overall percent germination, and overall root length. While not always statistically significant from the control group, this may give credence to previous research (Janeczko and Skoczowski 2005) conducted which demonstrates that most species have augmented reactions to low concentrations of β-estradiol, while high concentrations cause inhibitory effects.

Different study performed on *Zea mays* L. with conflicting data.

When the above set of experiments was started there had been no other previous study that investigated the effects of β-estradiol on *Z. mays*. It should be noted that during the course of experimentation and data collection a study was published that investigated the effect of β-estradiol on the germination of corn kernels (Erdal et al. 2011). The experimental procedures as well as concentration of β-estradiol used in this study were vastly different than those reported in this thesis study, which might explain

the variance in results between the two. In the study by Erdal et al. (2011) the *Z. mays* kernels were only exposed to the hormone for six hours in concentrations of 10^{-4}, 10^{-6}, 10^{-9}, 10^{-12}, and 10^{-15} M before they were placed into a sterile environment with 10 ml of distilled water. The steroid used in both studies did come from the same manufacturer.

In the study by Erdal et al. (2011) they found that all concentrations of β-estradiol significantly increased the kernel germination rate when compared to the control. However, by the end of the 5^{th} day all treatment groups as well as the control group had reached 100% germination. In their study they also found that the root and coleoptile elongation were significantly stimulated in the presence of the hormone, with the optimal treatment groups reported at 10^{-9} and 10^{-12} M. Their final conclusions were that the hormone β-estradiol (at the concentrations used) augmented germination velocity, as well as the length of the root and shoot. Erdal et al. (2011) do note that in studies on other plant species it has been reported that low doses of the hormone may enhance germination, while often times higher doses reduced or prevented it. This may be part of the difference between the results presented in this thesis and the studies by Erdal et al. (2011). Not only were the concentrations of β-estradiol used in the current study vastly different, but the length of time the kernels were exposed to the hormone in this thesis was 5 days, not six hours.

Implications of this current experiment

Mammalian sex hormones are constantly excreted into the environment through the urine and feces of mammals. A woman naturally can excrete around 10 µmol per day (Shore et al. 1993). The amount excreted in the urine increases with the intake of birth control pills and during ovulation. Chicken manure can contain over 1 µmol of estrogen in every gram (Shore et al. 1993). Larger mammals such as cows, horses, and swine can naturally excrete much larger amounts every day. When exposed to the environment either directly or through washing techniques and manure spreading, the estrogen in the excrement gets transferred into the soil as well as gets carried into various water sources. Several studies by independent researchers as well as the Environmental Protection Agency (EPA) have shown that estrogen is a persistent chemical in the environment and various water sources (Shore et al. 1993; United States Environmental Protection Agency, 2012). Mammalian sex hormones have been found in both lake sources as well as sewage water sources, some of which are used for irrigation. Both sources contain hormone concentrations that have been shown to affect plant growth and development (Shore et al. 1993).

It should be noted that there have been studies conducted on the ability of specialized sewage sludge bacteria to degrade β-estradiol and its metabolites into a non-estrogenic form. Through a proposed series of chemical reactions estradiol is degraded by bacteria into tricarboxylic acid (Lee and Liu 2001). In one study it was shown that aerobic degradation by activated sludge (sewage that is aerated and

contains aerobic bacteria) was able to remove about 88% of the estradiol. However, the anaerobic sludge bacteria were only able to degrade 50% of the hormone (Lee and Liu 2001). It was also noted in this same study that the type of sewage treatment had an effect on the ability of the bacteria to degrade the hormone. It was shown that activated sludge was much more efficient at degrading β-estradiol than a trickling filter system (Lee and Liu 2001). The conclusion was drawn that the vigorous mixing and aeration, as well as the high levels of microbial activity found in the activated sludge techniques, was the reason for the higher efficiency of degrading natural estrogens (Lee and Liu 2001).

How estrogens in water gets into field crops

Not all estrogen contaminated water undergoes treatment in waste water facilities. Much of the estrogen contamination occurs when runoff from farms and livestock facilities goes into natural water sources such as rivers and streams. These water sources can then be applied directly to fields through irrigation techniques or even periodic flooding. Once the β-estradiol is in soil, the crops can readily take up the hormone along with other nutrients and ions when water is taken into the root tissue through the root hairs. The water and dissolved nutrients and ions then traverse the dermal and ground tissue finally entering the xylem tissue where they become xylem sap. The process by which water is transported through a plant's tissues is known as cohesion-adhesion-tension theory of water movement (Taiz and Zieger 2006). Once

within the cells of xylem tissue (tracheids and vessel elements), the water molecules experience a pull upward through the tissue, the driving force being transpiration or loss of water vapor from the stoma of leaves. The water molecules each exhibit an unequal sharing of electrons causing a partial positive and partial negative charge across each molecule. These partial charges then form hydrogen bonds with neighboring water molecules in a property known as cohesion. The molecules also adhere to the surface of the xylem tissue through these partial charges and this is known as adhesion. The cohesion-adhesion-tension theory of water along with the process transpiration is considered to be the primary mechanism by which the xylem sap moves upward against gravity and into the shoot system of plants. Another property of water is its ability to be a universal solvent. This property allows water to dissolve many different substances, and then transport and cycle them through organisms' tissues as well as through the environment (Campbell et al. 2011).

Possible effects on staple grain crops and consequences

Farmland across the Midwest portion of the United States is typically dominated by soybeans (*Glycine max* (L.) Merr.) or field corn (*Zea mays* L.). A large portion of farmland is also dedicated to wheat (*Triticum aestivum* L.) and sorghum (*Sorghum bicolor* (L.) Moench) production. Corn, wheat, and sorghum are all staple grain crops in the United States. Their production has an enormous economic impact. In the U.S. the economic value of corn is at an all-time high and was recorded to be around two billion

dollars in 2010 (University of Missouri Extension, 2012). If the germination, growth, or overall yield of these staple crops are being affected by β-estradiol contamination of water sources this could have serious consequences on a global economic scale. In this study it was concluded that high doses of the hormone had a negative impact on *Zea mays* L. kernels and seedlings in several of the parameters tested including germination percentages and root development.

Further tests should be conducted to follow the development of the seedlings through maturation and the production of fruit to determine if the high doses of β-estradiol affect the later stages of development as well. A field study might also be beneficial for this area of research. So far, previous studies as well as this current study have all looked at the effects of β-estradiol on germination and growth in sterile laboratory conditions. However, as stated above there are bacteria that can degrade the hormone into different conjugates and non-estrogenic forms (Lee and Liu 2001).

The basic methodology described in this thesis study also needs to be continued on with field studies. The mass production of corn, sorghum, and wheat does not occur in a laboratory, but out in a field exposed to its biotic and abiotic surroundings. These grain crops are harvested for livestock feed and silage as well as human consumption and it would be important to understand the effects β-estradiol might have on overall production. Researching growth in the field would also take into account the presence of soil bacteria and natural climate conditions as well. Therefore that is one direction

future studies should take in order to determine how much of an effect β-estradiol has on corn where the actual growth of these plants takes place.

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Acknowledgements

I would like to first and foremost thank my thesis committee for their long hours and multiple revisions of this body of work. Dr. Karen Schaffer, I thank you for coming up to me at the vending machines in Garrett-Strong and asking if I had any research projects in mind. If it hadn't been for you, the ball never would have started rolling on this project. Dr. Jeff Thornsberry, I thank you for your knowledge of corn and your help with my experimental design. I also thank you for always answering my questions regarding all things maize. Dr. Ahmed Malkawi, I thank you for your knowledge of chemistry and the inner workings of molecules and their interactions, especially when it came to dissolving estradiol.

I gratefully acknowledge Dean Gregory Haddock and the Northwest Missouri State University Graduate School, Dean Charles McAdam and the College of Arts and Sciences and the Department of Natural Sciences for the funding of my research as well as the lab space and use of equipment. I would also like to thank Dr. Mark Corson for allowing me to work with Northwest Missouri State University while completing this body of work. Your continual support and commitment to my completion has truly been a gift.

For the rigorous and challenging comprehensive exam questions that allowed me to broaden my knowledge and really think about the writing process, I must thank Dr. Peter Adam. Even though I hated those five behemoths you called short essays, they were absolutely essential in this process.

Dr. Gregg Dieringer and David Vlieger, I thank you for your involvement and for teaching me the innerworkings of SAS statistical programming. Your teachings made the analysis of my results easy to work with and present to the public.

The following people must be commended for their continuous encouragement and support of this ongoing and strenuous project: Lisa Crater, Alex Bolick, Samantha Barton, and Shannel Phillips. Your ability to discourage my procrastination habits was truly a gift and I thank you every day for your involvement as well as for keeping me sane. To the countless other people involved in this journey, I must also give thanks. Your involvement, no matter how minor, will never be forgotten.

The biggest thanks of all must go to my parents David and Cynthia Bowlin. Your countless hours on the phone, encouragement, support, and questions have all led to this completed body of work. This is the culmination of not only my hard work, but yours as well. For you are the ones who have always pushed me to do my best and to always strive for success in all that I do. You both mean the absolute world to me and I cannot thank you enough. I know that you will smile as I do when I say, “I finished!”