EXPLORING GENETIC DIVERSITY AND ADAPTIVE PHENOTYPES
OF A WILD, PERENNIAL SUNFLOWER, HELIANTHUS CUSICKII
FOR CROP UTILIZATION

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Gina Maria Sideli
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Perennial grain crops are presently offered as a solution to current agricultural problems such as soil erosion, excessive water use, and water quality degradation while supporting ecosystem services. A longer growing season allows for a substantial root system to develop for the assimilation of nutrients and water at greater depths, minimizes nitrogen loss and conservation of water. A wild, perennial sunflower, *Helianthus cusickii*, close relative to the domestic sunflower, *Helianthus annuus*, has the potential to offer beneficial alleles for crop improvement to *H. annuus*. In particular, *Helianthus cusickii* could be a source of genes for drought tolerance, since it is found on dry, rocky sites in low rainfall regions and has a distinctively robust taproot. Here I evaluate the agronomic value of *H. cusickii* and whether it merits further research in sunflower crop improvement. Specifically, I characterize genetic variation via microsatellites within and among
populations of *H. cusickii* and phenotypically assess important, agronomic traits in field trials. A study of nine populations demonstrated *H. cusickii* had a considerable amount of diversity and low population structure with $H_e = 0.47$ and $F_{ST} = 0.247$. Growth performance studies showed that *H. cusickii* did not adapt well under field conditions in southern Kansas during two field seasons. These results did indicate that *H. cusickii* had greater vigor and survival in an unirrigated field with earlier planting, rather than an irrigated field with later planting, however this also resulted in smaller sized plants. Comparison of annual and perennial root traits showed an average root:shoot ratio of 0.735 in *H. cusickii*, and an average root:shoot ratio of 0.442 in *H. annuus*. Interspecific crosses (*H. cusickii* x *H. annuus*) between 23 plants resulted in the formation of 20 seeds, however plants grown from the hybrid seed did not reach anthesis.

I conclude, based on these experiments that *H. cusickii* holds potential for use of donor genes in domestic sunflower cropping systems. As this research served as a preliminary analysis of this wild perennial sunflower, further studies would be needed in order to assess plant water relations and the most beneficial quantitative trait loci.

Keywords: *Helianthus annuus, Helianthus cusickii*, phenotyping, genetic diversity, germplasm-bank accessions, microsatellite markers, perennialization
CHAPTER I

INTRODUCTION

Modern Agriculture Issues

The human global population is projected to reach nine billion within 50 years which will intensify the demand for food despite the fact that land and water resources are not projected to also increase, and may even decrease (Godfray et al. 2010). Urbanization and land degradation counterbalance expansion of agriculture into forest and grasslands, potentially leaving less farmland on which to produce food (Tanksley and McCouch 1997). Even more, there has been an increased emphasis placed on inputs such as chemical fertilizers, pesticides, and water use in crop production (Cassman and Wood 2005). The way food is produced, stored post-harvest, and distributed must be evaluated carefully to maximize the most efficient use of resources (Godfray et al. 2010).

Furthermore, global food supply must double in the next 25 years in order to keep up with the population increase (McCouch et al. 2013). New perennial grain crops on large acreages could resolve several agriculture issues. Research into these new crops could also provide ways to improve current annual crops.

Agriculture is one of the largest human disturbances (Glover et al. 2009). About 30% of the world’s total terrestrial land area is allocated to agricultural crops or cropland pastures (Scherr and McNeely 2008). As a result of modern agricultural practices, environmental problems have the capacity to decrease agricultural productivity
at varying levels, and even interfere with ecosystem services (Hazell and Wood 2008). Of the largest concerns are excessive water use, water quality degradation, nitrogen loss, animal and plant biodiversity loss, soil erosion and soil contamination (Cassman and Wood 2005; Zhang 2011).

Water quality and available water resources can be compromised by agriculture. Surface or ground water is used to irrigate fields resulting in polluted waters, which reduces the amount of water that is available for other purposes (Horigan 2002) such as urban and industrial use, hydropower plants, recreational use, freshwater fisheries and protection of natural ecosystems (FAO and PAR 2010). In many countries 50% of water is used for irrigation (Bennett 2000; Prathapar 2000; Qadir et al. 2003), and sometimes even 90% of total water use (Allan 1997; Qadir et al. 2003). As the demand for water increases with agricultural need, water shortages lead farmers to produce more even more with less irrigation water (Hazell and Wood 2008). Sediments from agricultural runoff flow into surface waters, which alter the water quality. Additionally, irrigation water leaves behind salts in soil and over time the salts decrease the soil's productivity (Horigan 2002). Water contamination can also occur from the overuse of chemical fertilizers and pesticides used in annual cropping systems. During the “Green revolution”, from the 1960s through 70s, improvements in plant varieties dramatically increased grain yield but also resulted in an increased usage of chemical fertilizers and irrigation. The green revolution resulted in higher crop yields in Asia and Latin America, yet led to an increased reliance on only a few high producing varieties which required mechanical harvest (IFPRI 2002). Consequently, the over application of fertilizers has
led to environmental contamination of water supplies in developing countries (NRC 1989). However, due to environmental awareness and implementation of policy, America and Europe have made efforts to reduce the amount of applied chemical fertilizers after accounting for 80% of worldwide usage in 1997 (FAO 2003). Moreover, the mismanagement of fertilizer application contaminates groundwater, leaves downstream agriculture affected and municipalities with additional expenses for water purification (FAO and PAR 2010). Today, only 30–50% of applied nitrogen fertilizer (Smil 1999; FAO and PAR 2010) and ~45% of phosphorus fertilizer (Smil 2000) is taken up by crops and the remainder can be lost by runoff and erosion from water moving across or in soil, leaching, harvesting, volatilization or denitrification. Organic systems of annual crops do not solve the issue of inefficient water and nutrient uptake due to small rooting systems (Cox 2010).

The loss of nitrogen from annual crops is estimated to be 30 to 50 higher than that from perennial crops (Randall and Mulla 2001; Cox et al. 2006). Application and runoff of nitrogen and phosphorus fertilizers into surface waters can lead to eutrophication and further cause algal blooms, which can lead to destruction of fish and aquatic flora. Ecosystem nutrient cycles are adversely affected, with increased usage of chemical fertilizers and pesticides (Tilman et al. 2001; Cassman and Wood 2005; Cox et al. 2006). Therefore, without breakthroughs in plant water-use efficiency, agriculture production will be unable to expand (FAO 2003).

Soil erosion caused from tillage of annual cropping systems can negatively affect crop yields. Tillage has been used for centuries to suppress weed growth, however
subsequent to machine degradation, the soil is compacted and left bare, vulnerable to erosion by rainfall and wind (Morris et al. 2010). Soil compaction then makes rooting of plants difficult, decreases soil water holding capacity, reduces soil organic matter, and alters soil characteristics (Pimental et al. 1995; Lal 1998; den Biggelaar et al. 2001; Pimental et al. 2012).

Agriculture depends upon diversity, yet is a big contributor to biodiversity loss. Decreases in biodiversity can occur at varying scales, and can affect both plants and animals (Tilman et al. 2001; Cassman and Wood 2005; Cox et al. 2006). Agricultural monocultures replace biodiversity among native landscapes and increase crop susceptibility to disease. Genetically uniform crops utilized in modern agriculture do not have the diversity necessary to protect against fungi, bacteria, viruses, arthropods and weeds (Cox 2002). For example, during the 1970s, southern corn blight drastically reduced corn yields in the United States. Susceptibility to the southern corn blight was found to be linked to a male sterility gene that was introduced into corn varieties (Ullstrop 1978; Tanksley and McCouch 1997). Agriculture depends on the development of novel plant varieties and crop diversification in order to withstand evolving plant diseases (Horigan 2002).

Industrial agriculture favors a monoculture system for highly productive varieties that are also ideal for mechanical harvest due to their uniformity. Unfortunately, the overdependence on genetically uniform crops has led to the loss of within-species diversity. The FAO (2011) estimates that there has been almost 75% of the genetic diversity loss in agricultural crops since the beginning of this century. However, the
introduction of genetic diversity from crop wild relatives can mitigate this loss. Undomesticated plants have adaptations that have aided their survival under harsh environmental conditions, but few studies have examined their adaptive potential under field conditions (McCouch et al. 2013). Therefore, it is imperative that agricultural scientists analyze the phenotypes and adaptive performances of the crop wild relatives (McCouch et al. 2013). Addressing these environmental issues associated with agriculture will be necessary to produce an adequate food supply (Tilman et al. 2002). Organic agriculture is expanding due to its focus on improving biological and human health (Cox 2010). However, ultimately a sustainable production system that has the ability to produce high yielding plants continuously, still support ecosystem services and reduce environmental impacts is needed (Glover et al. 2009).

Perennial Grain Crops: A Solution to Agriculture Issues

Perennial grain crops have been introduced to offer a solution to our current agriculture problems by providing benefits to society, farmers, and ecosystems more effectively than annual crops, which require demanding energy inputs that can be detrimental to the environment (DeHaan et al. 2005). Longer growing seasons allow perennial crops to develop a substantial root system that assimilates water and nutrients at greater depths than annual plants (DeHaan et al. 2005; Zhang et al. 2011), and the possession of this root system can potentially address the hazard of water and soil contamination. In addition, perennial crops minimize nitrogen loss in soils, reduce soil erosion because no tilling is required, provide a continuous habitat for wildlife so that
biodiversity is not reduced, and sequester carbon from its sustained root biomass (Zhang et al. 2011). Furthermore, most of the world’s landscapes are perennial plants, but only annual crops are used in more than 2/3 of worldwide cropland (Chiras and Readongk, 2004; Cox et al. 2006). The implementation of oilseed and grain perennials can secure the global food supply.

Perenniality Defined

Perenniality in plants may result from several vegetative organs such as root structures, rhizomes, stolons, tubers, or taproots (Hu 2003). Perennial species can be woody shrubs or trees that live for more than two years or herbaceous plants that have a substantial root system. Their reproductive life strategies differ from annual species in that many perennials have long juvenile phases, allocating much energy to reproduction by stolons or rhizomes and can reproduce both sexually and asexually (Petit and Hampe 2006; Savolainen et al. 2007; Smith and Donoghue 2008; Vallejo-Marín et al. 2010; Miller 2011). Many perennials have been domesticated as global food commodities (Schreckenberg et al. 2006). These include citrus fruits (oranges *Citrus sinensis* L. and lemons *Citrus limon*), vine fruits (table and wine grapes *Vitis vinifera*), dried fruits (almonds *Prunus dulcis* and pistachios *Pistachia vera*), young asparagus shoots (*Asparagus officinalis altilis*), and tubers (jicama *Pachyrhizus erosus* and Jerusalem artichoke, *Helianthus tuberosus*). Some perennial plants such as coconut *Cocos nucifera* L. or apple *Malus domestica* Borkh. are very high yielding, however they are not a viable substitute for annuals grains because these are more difficult to harvest (Van Tassel et al. 2010).

There are two approaches in breeding for perennial grain crops. The first is by direct domestication in which a wild species undergoes recurrent cycles of artificial selection for desired traits (Cox et al. 2006). This process can be laborious, taking many generations of selective breeding. The second approach is through hybridization of a target crop with a perennial wild relative, also followed by artificial selection (Cox et al. 2006). A major advantage of utilizing interspecific crosses over direct domestication is that a part of the domesticated genome is also found in the hybrid, thereby making selection of existing domestication genes possible (Kantar et al. 2013).

Although there have been useful cultivars produced in past research, breeding barriers arise from combining genomes of annual cultivars with perennial wild relatives (Jones et al. 1999; Reimann-Philipp 1986; Waggoner 1990; Sacks 2003). Problems were
first documented with the hybrids of wheat (annual) x wheatgrass (perennial) created during the 1920–1980 where hybrid crosses were found to be meiotically unstable, unfertile, and had unusual agronomic traits such as small seed size, tendency to lodge, reduced winter-hardiness and high mortality rates (Hayes et al. 2012). Whelan (1978) examined interspecific hybrids between Helianthus annuus and H. gigantus that had many abnormalities in the first generation, and concluded that despite both having the same number of chromosomes, the parents had a different genomic structure (Jovanka 2004). Meiotic abnormalities occur during meta-, ana- and telophases, which can lead to infertility, reduced pollen viability of F1 hybrids or even abortion of the hybrid embryo (Jovanka 2004). These genetic barriers can make the introgression of genes associated with perenniality root traits, cold, heat or drought tolerance and defense to pests very difficult (Hu 2003). As a first step, embryo culture rescue can be performed in order to overcome post-zygotic hybridization failure between species (Chandler et al. 1986). This is an in vitro technique in where a young embryo is placed on an artificial nutrient medium, which creates an optimal environment for growth and a substitute for endosperm (Reed 2005). It also can promote breaking seed dormancy.

The direct domestication approach skips these issues, has potential to introduce an alternative crop into agriculture production and further offer crop diversity. Both methods however need to demonstrate usefulness and economic acceptance (Hu 2003); however there are various trade-offs that need to be addressed such as abatement in production inputs versus reduction in yields from perennials (Sacks 2003).
The Sunflower Crop, a Candidate for Improvement

Sunflower is one of the four most important oil crops worldwide (Putt 1997; Burke et al. 2002) and is projected to expand in land use in developing countries (FAO 2003). It is widely used in products such as birdseed, confection seed for humans, and vegetable oil. The oil is a high quality, edible oil (no trans-fats and high in vitamin E) and can also be used to make biodiesel fuel (Jan and Seiler 2010; Kantar et al. 2013). Thus, the sunflower is an ideal candidate for the introduction of beneficial drought tolerance alleles or perennialization to improve its sustainability in agricultural production.

The *Helianthus* genus contains 50 species of annual and perennial plants native to North America (Heiser 1969; Rieseberg and Seiler 1990). Wild sunflower species have been widely studied, and have contributed novel genes and desired characteristics for crop improvement (Thompson et al. 1981; Seiler 1988a; Seiler 1992). Its genome is currently being sequenced via the Compositae project, which is a collaboration between by scientists at several universities in North America including the University of British Columbia, University of California-Davis, California State University-Pomona, University of Massachusetts-Boston, University of Georgia, and Indiana University. This project aims to create gene libraries and genetic maps for agronomically important traits of economically important species, and enhance the ability for introgression of crop wild species.

A wild, perennial sunflower, *Helianthus cusickii* (Figure 1), is a close relative to the domestic sunflower, *Helianthus annuus* (Timme et al. 2007). It is in fact more
closely related to the annual crop sunflower than the other perennial *Helianthus* species being used in perennial oilseed research at The Land Institute and the University of Minnesota. *Helianthus cusickii* belongs to the subclade Pumili, within the Ciliares clade, and is noted for morphologically being the most extreme species in the genus (Heiser,
Helianthus cusickii is native to western United States (i.e., Arizona, Oregon, Washington, Nevada and California) and grows and reproduces in dry, rocky soils of mountain deserts, surviving with an annual precipitation of merely 25-60 cm (Seiler 1992). The flowering time of *H. cusickii* (May - July) differs from *H. annuus* (August - October). Interestingly, the oil content of *H. cusickii* seed ranges from 220-307 g k$^{-1}$ with an average of 263 g k$^{-1}$; by comparison wild *H. annuus* has average seed oil content of 290 g k$^{-1}$ (Marek et al. 2005).

*Helianthus cusickii* exhibits interesting traits related to drought tolerance and was selected for this study based on its root phenotype, extreme adaptation to limited water availability, and close relationship to *H. annuus*. Morphology: it is less than one meter tall, produces a basal rosette of leaves, dense pubescence on both leaves and stems, and possesses perenniality from a taproot. The leaves of *H. cusickii* are petiolate, opposite, and lanceolate or falcate (hook or sickle) shaped, with undulating and entire leaf margins. *Helianthus cusickii* has glandular trichomes on its leaf underside, which contain sesquiterpene lactones, known to contribute to plant defense from insects (Picman 1986; Timme et al. 2007). To date, there has not been genetic and phenotypic analysis of *H. cusickii*.

The Role of Germplasm Banks in Facilitating a Solution

Genetic resources are key to increasing food security, while improving human and ecosystem health (FAO and PAR 2010). Maintaining the diversity of genetic
resources is the foundation for successful breeding programs (Terzic 2012). A principle mission for plant breeders and geneticists is to first discover related species that contain diversity, and utilize their genetic resources for crop improvements (Varshney et al. 2008; Huang 2010). For example, crop diversification between domestic and wild relatives can limit the effects of disease, or enhance the crop’s ability to yield under poor soil conditions through the introgression of resistance or beneficial genes.

Germplasm or seed banks are a valuable resource of genetic variation (Tanksley and McCouch 1997). If a crop wild relative is regarded a useful genetic source for crop improvement, the first task is to determine the genetic variability within and among its populations (Chann et al. 1997). It is expected that wild species contain high levels of variation due to low selection pressures (Chann et al. 1997), and the genetic diversity, contained in wild species can help to advance crop improvement. For example, a wild species of rice, *Oryza nivara*, found after screening of over 6,000 seed-bank accessions, contains resistance genes against grassy stunt virus in Asia. Crossbreeding with domestic rice resulted in the transfer of this resistance (Plucknett et al. 1987; McCouch et al. 2013). Furthermore, the maintenance of seed banks provide a way to preserve wild species populations and future utilization of plant genetic resources (Seiler 1992).

**Molecular Markers Used in Genetic Diversity Studies**

To date there are many molecular strategies employed to assess genetic diversity in germplasm accessions. Neutral molecular markers are advantageous because
the environment does not influence them, and genetic diversity can be ascertained without prior pedigree information (Bohn et al. 1999; Kuleng 2006). For the purpose of characterizing germplasm, DNA markers have been widely utilized in order to infer genetic distances, evaluate genetic diversity and further provide valuable ecological and evolutionary information (Hoisington 1999). The assessment of genetic diversity addresses the identity, relationship, and structure among individuals, population accessions, and related species (Westman and Kresovich 1997; Dje et al. 2000). Among these methods are restriction fragment length polymorphism (RFLPs), single nucleotide polymorphisms (SNPs) and microsatellites (SSRs). Microsatellite markers have the ability to distinguish different genotypes (Yang et al. 1994; Russell et al. 1997; Bredemeijer et al. 1998; Dje et al. 2000).

Microsatellite Markers

Microsatellites are tandem repeat sequences of short unit DNA motifs that are located in or near introns, which contain variability in the number of repeats per locus (Temnyk 2001). They are popular due to their co-dominant expression, being linked to a locus, which then enables the discrimination of homozygotes and heterozygotes (Clement et al. 2010). SSRs are widely available and have extensive allelic diversity that can identify different individuals and genetic population structure. Here, the genetic sequence is not directly analyzed, but rather alleles are examined through genetic markers for a specific locus. Microsatellite markers are commonly used in genetic diversity studies for many species such as bean Phaseolus vulgaris L. (Burle 2010), sorghum, Sorghum bicolor L. (Dje et al. 2000), wine grape, Vitis vinifera L. (Emmanuelli et al. 2013), rice,
*Oryza sativa* L. (Liakat Ali et al. 2011), and *tef, Eragrostis tef* Zucc. (Zeid et al. 2012). Gevaert et al. (2013) investigated a rare, endemic sunflower *Helianthus porteri*, using SSRs to determine genetic variation and population structure. They concluded that this sunflower had unexpectedly high genetic variability for a rare species and low population structure. The employment of SSRs across a plant genome is a key tool for plant breeders to determine markers that may be useful in the identification of quantitative trait loci and whether species contain sufficient genetic variation for crop improvement (Dje et al. 2000). Specifically, expressed sequence tag EST-SSRs are highly conserved and transferrable between related species due to their location within genes. In addition, it has been proven that EST-SSRs produce an easier data output to interpret because are located within coding regions and have a reduced occurrence of null alleles (Ellis and Burke 2007).

**Objectives**

I am assessing the value of *H. cusickii* as a source of genetic variation for crop improvement, and whether this variation justifies its utilization in the development of a perennial crop sunflower. I will accomplish this by evaluating genetic diversity and population structure of *H. cusickii* germplasm and phenotypically assessing agronomical traits related to drought resistance and adaptive growth performance under field conditions.
Questions

1. How much genetic diversity does Helianthus cusickii have, and how is it partitioned among and within populations?

2. Does H. cusickii exhibit novel agronomic traits and adaptability when transplanted to the agricultural environment?

3. What is the best growth regulator to break H. cusickii seed dormancy?

Hypotheses


2. The possession of drought tolerance or perenniality traits in Helianthus cusickii might be useful in agriculture production in order to support its adaptability to specific field conditions.

3. Ethylene treatment will break seed dormancy faster than other growth hormones.
CHAPTER II

MATERIALS AND METHODS

Genetics Approach

Plant materials, DNA Extraction and Genotyping of Samples

A total of 17 H. cusickii germplasm accessions (Appendix A), hereafter named populations, were obtained through United States Department of Agriculture Germplasm Resource Information Network (North Central Regional Plant Introduction Station, Ames, IA). Sampled populations ranged in size from 50 to > 1000, and an equal number of heads per plant were collected at random within a population and pooled. When population size was smaller, sampling was taken from each plant (Marek personal communication). Seeds were placed in trays with moistened filter paper and put in a cold room at 3-5°C for a period of 56 days. Trays were removed from cold room and placed at 20-24°C. After successful germination, seedlings were transplanted into a two field plots at the Land Institute in Salina, Kansas containing New Cambria soil, a class I soil, and reared in two different growing seasons (2012, 2013).

Nine H. cusickii populations were used in the genetic diversity study (Figure 2) and population sampling ranged in size from 13 to 20 plants. Total genomic DNA was isolated from young tissue using 100 – 200 mg of fresh leaf samples that were frozen in
Figure 2. Location of nine native *Helianthus cusickii* populations. Each are represented by the last three digits of PI number, and were used for genetic diversity study. Seeds were obtained through USDA Germplasm Resource Information Network.

liquid nitrogen, ground with a mortar and pestle and processed using Qiagen DNeasy plant mini extraction kit following manufacturer’s instruction (Qiagen, Valencia, CA).

Microsatellite or SSR (simple, sequence repeat) marker analysis was accomplished at GenoSeq Center, University of California, Los Angeles in order to
evaluate genetic variation within and among *H. cusickii* populations. A total of 163 DNA samples were genotyped using 14 expressed sequence tag, EST-SSR genetic markers (Table 1) initially developed for *H. annuus* (Tang et al. 2002) with successful amplification in other *Helianthus* species (Ellis et al. 2006; Pashley et al. 2006; Gevaert et al. 2013; Mandel et al. 2013). Primers were fluorescently labeled with FAM, NED, VIC or HEX (Life Technologies, Carlsbad, CA). PCR was performed in a total of 12.51 ul containing 10 ng/ul of DNA, 5x Expand long range PCR buffer with 12.5 mM MgCl₂, 10 uM primer mix, DMSO, 10 mM dNTP, Expand Long Range Enzyme mix 5U/ul.

Roche Expand Long Range dNTP pack (Life Technologies, Carlsbad, CA) was used for PCR with 25 ng of template DNA. PCR was performed on ABI9700 dual block PCR machine. PCR conditions were as follows: 92 °C for 2 min, 92 °C for 10 sec, 60 °C for 15 sec down by 0.5 °C every cycle, 68 °C for 30 sec, repeat 20 times, 92 °C for 10 sec, 52 °C for 15 sec, 68 °C for 30 sec, repeat 25 times, 68 °C for 7 min, 10 °C forever.

Multiple PCR products were pooled 1:1:1 and 3 ul of the pool was loaded onto ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA) with GeneScan LIZ500 (Life Technologies, Carlsbad, CA) as a dye size standard and run for fragment analysis. Genotyping was done using Genemapper V4.0 software (Applied Biosystems, Foster City, CA).

**Population Genetic Analyses**

Indicators of genetic diversity were estimated for nine *H. cusickii* populations, including mean number of alleles, alleles per locus, observed heterozygosity, Nei’s
Table 1. Primer information for 14 EST-SSR markers used in this study. Markers derived from Pashley et al. 2006. Core primer nomenclature ID from Ellis et al. 2006.

<table>
<thead>
<tr>
<th>Core Primers</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Repeat motif</th>
<th>Expected size (bp)</th>
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<td>292</td>
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<td>ATGATGGAGCCACCTATGGA</td>
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<td>305</td>
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<td>BL0005</td>
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<td>253</td>
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<td>TGACACACAAACACCTTGC</td>
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<td>270</td>
</tr>
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<td>BL0017</td>
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<td>CAATACTACATCATATAATCGACCAAC</td>
<td>(tga)4</td>
<td>174</td>
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<td>BL0030</td>
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<td>GCATCATCCAAACAACTAGAAGG</td>
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<td>235</td>
</tr>
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</table>
unbiased genetic diversity, percentage of polymorphic loci, and within population inbreeding using GenAlEx 6.5.1 (Peakall and Smouse 2006). A Hardy Weinberg Equilibrium (HWE) chi-square test was performed to evaluate significant departures at marker loci. Isolation by distance was performed using a Mantel test implemented in GenAlEx (Peakall and Smouse 2006). Geographic data were gathered from GRIN accession database and a geographic matrix was created using Geographic Distance Matrix Generator V 1.2.3 (Ersts 2006; Mandel et al. 2013). Pairwise distance relationships of populations were compared using principal coordinate analysis, PCoA as implemented in GenAlEx (Peakall and Smouse 2006).

The Bayesian clustering program STRUCTURE (Prichard et al. 2000) was used to assess population genetic structure in all nine *H. cusickii* populations using 10 marker loci. Individuals from the total population were assigned to K = subpopulation clusters. Batch runs were performed for K = 1-12 population clusters with 5 runs per cluster. Initial burnin was set to 5,000 with 50,000 MCMC iterations. Based upon inferred clusters, another batch run was performed for K = 1-7 clusters with a burnin set to 50,000 with 100,000 MCMC at 15 runs per cluster. The use of log likelihood for each K, L(K), and ad hoc quantity, or DeltaK methods (Evanno et al. 2005) were used to find the true K. Data was plotted using STRUCTURE Harvester (http://taylor0.biology.edu/struct_harvest/; Earl 2011; Mandel et al. 2013). Population structure was also carried out via analysis of molecular variance, AMOVA, in GenAlEx 6.5.1 (Peakall and Smouse 2006) to estimate molecular diversity at each hierarchial level among and within groups for each marker locus wherein the significance for $F_{ST}$ was
tested at 999 permutations. The proportion of total diversity among populations was written as $F_{ST}$ (Wright 1951).

Growth Performance Studies

Phenotypically Assess *Helianthus cusickii’s* Adaptive Growth Performance in an Agriculture Field

Two distinct field experiments (A-2012, B-2013) at the Land Institute in Salina, Kansas were conducted in order to evaluate *H. cusickii* adaptive growth performance in an agriculture field (Figure 3). The environmental conditions were similar for both growing seasons, with respect to maximum and minimum temperature; however, there was significantly more rainfall in the 2013 year (Appendix B).

**Year 1, Field Plot A, Under Irrigation**

Five *H. cusickii* populations (Appendix A) were used in this experiment as they yielded greater than 22 seedlings after germination. Seedlings were transplanted into field plot A on the 25 of July 2012. The plot was irrigated 0.305 meters below the ground, and contained 198 *H. cusickii* plants that were grown one meter apart in single rows spaced 1.5 meters apart. Four blocks were established and 9 populations were randomly positioned within each block, with five replicates per block. When plants reached anthesis (77 days), the following traits were scored: quantitative (plant height, leaf length, peduncle length, floral head diameter, floral heads/plant) and qualitative (survival status, vitality, maturity). Qualitative measurements were ranked as follows: survival, 0 – dead or 1- alive; maturity, 1- leaves only, 2- flowers unopened, 3- flowers
with 50/50 opened to closed, 4- flowers 90/10 opened/closed, 5- flowers dried; vigor 0 -
dead, 1- mostly brown vegetation with some green, heads wilted (signs of weevil
damage), 2- green vegetation with some browning on leaves, heads upright, 3- all green,
no browning or herbivory.

Artificial crosses were carried out, as part of experiment A, between *H. cusickii* and *H. annuus* parental lines Triumph s668 (Dow AgroSciences LLC.) in order
to test the crossability between species. Heads of both species were bagged before anthesis. Each day, bags were removed and heads were inspected. As *H. annuus* heads began to shed pollen, the pollen was brushed into a jar and kept in a low-humidity refrigerator until needed. When anthesis was observed among florets on *H. cusickii* heads, *H. annuus* pollen was applied to those florets, using a brush, in the late morning or afternoon when the styles had elongated and the stigmatic surface was revealed. Emasculation was not performed because wild *Helianthus* species are known to be strongly self-incompatible (Burke et al. 2002). Followed by each pollination, heads were quickly rebagged to prevent pollination by insects. The same heads were pollinated on multiple following days as additional whorls of florets underwent anthesis. A total of 23 *H. cusickii* plants, arbitrarily selected, were used as female parents. *Helianthus cusickii* floral heads were left to develop naturally without the use of embryo rescue, or *in vitro* fertilization. After 14 days, bagged heads were collected and screened for viable first generation interspecific hybrid (F1) seed. Resulting seeds were weighed, measured and stored in a cool dry storage room.

**Year 2, Field Plot B, A Non Irrigated Plot**

Experimental field plot B followed a random plot design containing 334 *H. cusickii* plants, which represented 17 populations (Appendix A). Plants were grown in single rows spaced every one meter without the use of irrigation lines below soil. A border row of *H. annuus* was planted adjacent to *H. cusickii* plants in order to visually compare between species plant size (Figure 3). This growing season was chosen to begin
earlier than first year experiment, on the 10 of June 2013 in order to simulate H. cusickii’s natural growing season of April – August. Due to an earlier planting season, gathered phenotypic data varied from plot A. The following quantitative data were collected at the time of anthesis: leaf characteristics, percent leaf cover in a 15 cm x 15 cm quadrat (for size measurement), leaf size (l x w), peduncle and number of stems; the following qualitative measurements were collected: status, maturity, vigor, and flowering. No interspecific crosses were performed.

During H. cusickii’s second year growing season in plot A, surviving plants were phenotypically scored for plant height, leaf size, flowers/plant, peduncle, maturity, vigor, seeds/plant, seed weights. Plants were harvested above and below ground to further examine root traits (root length, root diameter measured every 5 cm, dry root weight, dry shoot weight).

Breaking Seed Dormancy in Helianthus cusickii Germplasm

In order to determine the best growth hormone to break stimulate germination of wild seed, an additional experiment was carried out during winter 2013 at California State University, Chico greenhouse. Seed dormancy was evaluated with the effect of two growth hormones, ethylene and gibberellic acid. Helianthus cusickii seeds and putative F1 hybrid seeds (H. annuus x H. cusickii made by Shiela Cox, Land Institute, Salina, KS) from experiment A, were treated with either 1 mM ethephon (1 or 2 % aqueous ethylene [Florel brand, Monterey Lawn and Garden, Fresno, CA]) or a 1 or 2 % aqueous gibberellic acid solution. The seeds were first soaked in a 3 % H₂O₂ solution for five
minutes in order to clean the seed coat. For a 24-hour period seeds were soaked in either the ethylene or gibberellic acid treatments. Whatman filter paper was presoaked with treatments, layered on 9 cm petri plates and labeled. Seeds were stratified on petri plates, which were sealed with parafilm, and placed in 4°C cold room for a period of 14 - 30 days. At 14-day intervals, plates were removed from the cold room, seed endosperms were nicked, and placed at 21°C. If no radicle emergence occurred within seven days, plates were put back in cold room for an additional seven days, then removed again. Days were recorded when radicle emergence occurred and germination began. Seeds were observed daily to replace moldy filter papers, and moldy seeds were rinsed with distilled water.

Germinated *H. cusickii* and putative F1 hybrid seeds were transplanted to 7.62 cm pots in a sterile medium (one part worm farm soil [0.37 cu m worm castings, 0.23 m coco coir, 0.18 m compost, 0.32 m 3/8 pumice, 27.22 g osmocote, 27.22 g calphos 0-3-0, 27.22 g dolomite 65, 27.22 g green sand, 27.22 g oyster shell, 176 kg #3 Monterey sand], one part pumice, one part decomposed granite, one part Turface MVP [Profile Products] [A calcined, non-swelling illite and silica clay]) and grown in a greenhouse with 16-hour daylight. Plants were watered every two days and given 59.147 mL of 12% nitrogen fertilizer every 14 days. A total of 60 surviving plants were transplanted to 3.875 liter pots with ProMix professional growing mix (ProMix HP) and vermiculite with a 5:1 ratio, and were placed outside under daily watering at Floral Native Nursery, Chico, CA. Observational growth data was recorded in order to evaluate the hardiest populations.
grown in pots. Pollen grain staining of putative F1 hybrids could not be performed because no flowering occurred within the experiment.

**Statistical Analysis**

All *H. cusickii* quantitative phenotypic data were analyzed through SPSS Statistical Software (Armonk, NY) using analysis of variance (ANOVA), in order to test significant differences among the means of each population accession. A random block design, univariate analysis was used to test if block had an effect on measured quantitative traits. In the genetics study, a two-factor ANOVA was also used to determine if population and locus had an effect on heterozygosity. Significance value was determined at $p < 0.05$. 
CHAPTER III

RESULTS

Genetic Studies

Genetic Diversity in *H. cusickii*

A total of nine *H. cusickii* population accessions were genotyped. Ten out of 14 microsatellite markers were successfully amplified and found to be polymorphic, while the remaining four primer pairs did not yield scorable amplicons. Total species diversity as measured by Nei’s (1978) unbiased genetic diversity, \( U_{He} \), across these populations was found to be 0.407 ± 0.031 and average of expected diversity across same populations, \( U_{He} \), was 0.393 ± 0.030. California populations 966, 968 were found to have the highest gene diversity \( U_{He} = 0.507 \), and one of the Oregon populations 659 was found to have the lowest gene diversity \( U_{He} = 0.261 \pm 0.091 \). Mean number of alleles per polymorphic locus was 3.330 ± 0.218. The average of \( F_i \), inbreeding coefficient, displayed a low probability of inbreeding 0.193 ± 0.039 (Table 2). The mean of polymorphic loci for all populations was 75.56% ± 4.44%. Washington population 658 had the highest number of heterozygotes, \( H_o = 0.465 \), and was completely outbred, \( F = -0.036 \). A two-way ANOVA, with population and marker locus as fixed effects, detected that population had a significant effect in heterozygosity values \( p = 0.019 \) as well as locus \( p \leq 0.001 \).
Table 2. Measurements of genetic diversity within population accessions. Based on 10 polymorphic EST-SSRs.

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<tr>
<th>Accession</th>
<th>GRIN</th>
<th>Location</th>
<th>% P</th>
<th>N</th>
<th>Na</th>
<th>$H_o$</th>
<th>$UHe$</th>
<th>$F$</th>
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<td>±0.752</td>
<td>±0.105</td>
<td>±0.108</td>
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<td>659</td>
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<td>±0.091</td>
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<td></td>
<td></td>
<td></td>
<td>±0.333</td>
<td>±0.096</td>
<td>±0.091</td>
<td>±0.077</td>
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<td>±0.619</td>
<td>±0.055</td>
<td>±0.080</td>
<td>±0.106</td>
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<tr>
<td>958</td>
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<td>OR</td>
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<td>±0.111</td>
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<td></td>
<td>±0.786</td>
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<td>±0.554</td>
<td>±0.089</td>
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<td>Total</td>
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<td>±4.44</td>
<td>±0.281</td>
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<td></td>
<td>±0.281</td>
<td>±0.218</td>
<td>±0.030</td>
<td>±0.031</td>
</tr>
</tbody>
</table>

Note: GRIN ID refers to National Germplasm Resource Network, Location is by state within the United States, % P polymorphic loci, N number of sampled individuals, Na number of different alleles, $H_o$ observed heterozygosity, $UHe$ Nei’s (1978) unbiased expected heterozygosity, F, inbreeding coefficient.
Sequence polymorphisms at marker locus (BL001, BL005, BL010, BL011, BL012) in populations 657, 658, 661, 960 indicate significant deviations from Hardy Weinberg equilibrium (Table 3). Oregon population 661 consistently showed deviations from HWE in 8 out of 10 marker loci. Washington populations 657, 658 and Nevada population 960 had multiple significant deviations from HWE. In contrast, Oregon populations 659 and 958 displayed monomorphic loci. Alleles from marker locus (BL004, BL005, BL020) were shared from gene flow due to $N_m < 1$, and marker locus (BL011) displayed little migration $N_m = 0.220$. All populations contained more shared alleles than private alleles (Figure 4). Private alleles were found at all 10 loci with BL030 having the most (15) and BL001, BL004 only having one. Eight private alleles were found at a frequency of 0.71 or greater, and two greater than 0.25 (BL012 at allele 265; BL030 at allele 238). Population 657 contained the highest number of different alleles (4.1), and highest number of locally common alleles (1.4). Populations 966, 968 had greatest heterozygosity values (0.493, 0.463).

**Population Structure**

The $F_{ST}$ value as estimated in AMOVA was 0.234 among all populations ($p \leq 0.001$). Values of mean $F_{ST}$ per locus ranged from 0.138 to 0.532 ± 0.037, with an overall mean of 0.247 ± 0.037, thus indicating weakly structured populations (Table 4). The correlation of genes among individuals over all populations, as measured by $F_{IT}$, was found to be 0.359, and within individuals, within a given population $F_{IS}$, was 0.163. The overall AMOVA indicated that there was 23 % variation distributed among populations, 13 % variation among individuals in the total population, and 64 % variation within
Table 3. Results from Chi-Squared test to Hardy-Weinberg Equilibrium. Marker locus is displayed vertically and populations are horizontally shown. *$P$<0.05, **$P$<0.01, ***$P$<0.001, m = monomorphic

<table>
<thead>
<tr>
<th></th>
<th>657</th>
<th>658</th>
<th>659</th>
<th>661</th>
<th>958</th>
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Figure 4. Allelic patterns measured across nine *H. cusickii* populations. Genetic diversity or *heterozygosity* is measured on the right, and mean values on the left as follows: $Na$ is no. of different alleles, $Na (Freq \geq 5\%)$ is no. of different alleles with frequency greater than 5%, $Ne$ is no. of effective alleles, $I$ is Shanonn Index, $No. Private Alleles$ is no. of unique alleles to a given population, $No. LComm Alleles$ is the no. of locally common alleles shared (freq $\geq 5\%$) found in 25% or fewer of populations, and 50% or fewer of populations.
Table 4. $F$-statistics for 10 microsatellite marker loci; $F_{IS}$ as measured by the average inbreeding coefficient within subpopulations, $F_{ST}$ fixation index among populations, and $F_{IT}$ given as total inbreeding coefficient.

<table>
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<th>Marker Locus</th>
<th>$F_{IS}$</th>
<th>$F_{IT}$</th>
<th>$F_{ST}$</th>
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<td>0.358</td>
<td>0.231</td>
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<td>Mean ± SE</td>
<td>0.173 ± 0.105</td>
<td>0.374 ± 0.089</td>
<td>0.247 ± 0.037</td>
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Individuals in populations. When considering regional data, as divided by state, there was only 10% variation among regions.

Pairwise $F_{ST}$ values ranged from 0.041 - 0.346 and values were significantly different from zero $p < 0.001$ (Table 5). Population 659 had the highest $F_{ST}$ values when paired with other populations. There was no supporting evidence for isolation-by-distance theory, which aims to find a correlation between genetic distance and geographic distance ($R^2 = 0.02199, y = 0.0001x + 0.2763, p \leq 0.110$).

STRUCTURE analysis identified four true clusters: cluster one contained Washington populations 657, 658, cluster two contained Oregon population 659, 661, cluster three contained Oregon population 958 and Nevada population 962, and finally
Table 5. Pairwise population $F_{ST}$ values. $F_{ST}$ values are displayed below the diagonal and geographic distances above. Colors are represented to emphasize highest differentiation in red, to lowest differentiation in blue.

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cluster four contained California populations 966, 968 and Nevada 960 (Figure 5). Principle coordinate analysis, or PCoA, confirms the cluster groupings found in STRUCTURE, however population 960 was found be along the same coordinate with populations 958, 962 primarily due to numerous shared alleles, rather than cluster with 966, 968 (Figure 6).

Figure 5. STRUCTURE plot from $K = 4$ population clusters. Each individual is represented by its own line and the percent it fits (scaled on y-axis) to a given cluster, or its shared alleles with another cluster.

Growth Performance Studies

Adaptive Growth Performance

Having a total of 198 $H.\ cusickii$ plants in plot A, 73 plants survived within one growing season, 23 survived after the first winter, and only six plants (from populations 659, 658) survived through a second year of growth. Of the 23 interspecific crosses performed ($H.\ annuus$ x $H.\ cusickii$), four female $H.\ cusickii$ plants produced 20 putative F1 hybrid seed. From a total of 334 $H.\ cusickii$ plants in plot B, 262 plants survived over the course of one growing season.
Quantitative Trait Measurements

Results from phenotypic variation using ANOVA demonstrated that plants grown in plot A exhibited strong evidence \((n = 53, df = 48, p = 0.015)\) for variation among population means for leaf lengths (Appendix E). After multiple comparisons test, population 658 leaf lengths were significantly different from population 659 \((p = 0.022)\). Results from plants grown in plot B showed that leaf lengths \((n = 334, df = 223, p < 0.001)\) were significantly different between populations, as were leaf widths \((n = 334, df = 223, p = 0.030)\). Main differences for leaf lengths were seen between population 657 to population 661 \((p = 0.011)\) and population 960 \((p = 0.009)\), as well as population 958 and 7 others populations including 960, 964, 965, 967, 968, 658, 661 \((p < 0.05)\). The block design implemented in plot A did not have an effect on quantitative traits measured. The most robust populations grown in plots are listed in Appendix F.
First year *H. cusickii* growth differed from second year growth in plot A. Average plant height in first year growth was $33.860 \pm 2.460$ cm and $59.433 \pm 5.030$ cm for second year growth. Average flowers per plant for first year growth was $12.860 \pm 1.630$ and $37.330 \pm 8.040$ in second year growth. Seed set was not accounted for in year one growth since perennials spend their first year establishing roots, however seed set for second year perennial growth ranged between 13 – 94 seeds per plant.

In comparison to *Helianthus annuus* and *H. maximilliani*, *H. cusickii* had smaller above ground vegetative structures and larger root structures (Table 6). For example, *H. annuus* has a leaf size of $\leq 317 \text{ cm}^2$, *H. maximilliani* $\leq 239 \text{ cm}^2$ and *H. cusickii* $\leq 37.200 \text{ cm}^2$. The average dry root weight for *H. cusickii* was $32.830 \text{ g}$. The
largest taproot for *H. cusickii* had a diameter of 5.828 cm at its base, 0.215 cm at 40 cm from its base, and a dry weight of 52 gm, which was up to 5 times heavier than *H. annuus* root dry weight (Figure 7). Note that these roots were excavated and fine roots were not calculated into total root depth and weight. In contrast, Goodman and Ennos (1996) found *H. annuus* to have a 0.387 cm root diameter at its base, 0.183 cm at 8 cm, and a dry weight of 11.200 grams. Average root:shoot ratio for *H. cusickii* was 0.735 compared to 0.442 for *H. annuus* (Table 7).

Figure 7. Dry specimen comparison between *H. annuus* and *H. cusickii* (right). Plants were excavated, placed in a drying room in order to remove moisture, and weighed.
Table 7. Measure of root and shoot data for *H. cusickii* and *H. annuus*. Includes dimensions and dry weights of second year growth for *H. cusickii*. Data for *H. annuus* was derived from Goodman and Ennos (1996).

<table>
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<tr>
<th>Accession</th>
<th>Root diameter in cm measured every 5 cm from base</th>
<th>Total Length (cm)</th>
<th>Branch</th>
<th>Root dry weight (g)</th>
<th>Shoot dry weight (g)</th>
<th>Root: shoot ratio</th>
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<td>0.442</td>
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</table>
Measures of maturity were different between plot A and plot B; plants grown in plot A matured faster, having a mean maturity score of 2.408 ± 0.119, *n* = 71, rather than 1.945 ± 0.069, *n* = 319, at the time of data collection. Vigor score in plants was also found to be different between the plots, with greatest plant vigor score of 1.959 ± 0.066 seen in plot B, rather than 1.444 ± 0.074 in plot A. Percent survival, as measured at the end of growing season, was greater in plot B, (262 plants) or 78.44 %, and only (70 plants) or 34.85 % in plot A (Figure 8). Twenty-three plants or 12.8 % survival rate was noted for *H. cusickii* plants in plot A after a complete year. It is also important to note that the amount of annual rainfall differed greatly between the two plots with year one having 34.392 cm and year two having 63.392 cm (Appendix B).

Figure 8. Comparison of the surviving *H. cusickii* plants in one growing season for irrigated vs. non irrigated plots. Plants were tallied as dead or alive at the end of the each growing season, and percent survivals were calculated.
**Greenhouse/Nursery Plants**

A total of 52 potted *H. cusickii* plants survived over one growing season with population 962 yielding the greatest number (14) of plants, and one putative hybrid. Populations 960 and 958 also had a large number (7) of surviving plants. Of the total 20 putative F1 hybrid seeds, three from population 958 germinated, however due to a thermostat error in the greenhouse, the seedlings dried out and only two survived. These two surviving putative hybrid plants never flowered in this experiment, but were morphologically similar to *H. cusickii* with a larger leaf size. After the completion of a second year growth, one putative hybrid survived, flowered, and possessed a perennial root system. The seven remaining hybrid seed from population 659 never germinated. Observationally, populations 958 and 962 demonstrated the highest vigor score (> 2) amongst potted plants.

**Seed Dormancy Study**

Among all hormone treatments there was significant evidence for differences in days for radicle emergence (*n* = 86, *df* = 71, *p* < 0.0001). Multiple comparisons test revealed that these differences observed (*p* < 0.02) were between all treatments except between 2% ethylene and 1% gibberellic acid (Figure 9). The 1% ethylene treated seeds had the slowest mean germination time of 51 days, in contrast to the 2% gibberellic acid treated seed, which had a mean germination time of 19 days.
Figure 9. Influence of two hormone treatments on radicle emergence. \( E \) = aqueous ethylene, 1%, 2%, and \( GA \) = gibberellic acid 1%, 2%.
CHAPTER IV

DISCUSSION

Genetic Diversity Study

The primary purpose of this study was to genetically characterize a select portion of *Helianthus cusickii* populations in order to determine its use for crop improvements. EST-SSRs have grown in popularity and are widely used in genetic diversity studies for domestic or wild species (Varshney et al. 2005; Ellis and Burke 2007). *H. cusickii* populations exhibited low genetic substructuring $F_{ST} = 0.247$, was found to have a similar pattern $F_{ST} = 0.253$ with herbaceous perennials (Hamrick and Godt 1992), and higher $F_{ST}$ than *H. angustifolius* perennial sunflowers $F_{ST} = 0.174$ (Ellis et al. 2006). The majority of the genetic variation was found between individuals from a subpopulation, and only a small proportion of additional differences were found between populations, thereby indicating limited population gene flow. Differentiation among each population can be explained by a recent population level divergence. The Wahlund effect states that when two or more populations have different allele frequencies there is a reduction in overall heterozygosity (Xu 2013). This is noted in a larger $H_e$ than $H_o$. Causes are most likely due to obstructed gene flow by mountain ranges followed by genetic drift, thereby further causing some level of divergence in subpopulations from the meta-population. Higher genetic diversity among the peripheral populations would further indicate a restricted level of gene flow from central populations. However,
California population 968, which was the southernmost population, contained alleles from four of the other populations indicating long distance gene flow. Oregon population 659, which is located the farthest eastward, is largely inbred and fixed for different alleles than other populations. The fact that regional or population variation is so low (about 10% from the AMOVA results) reflects relatively low structure across populations, and further reflects the poor correlation from the Mantel Test. Non-significant differences in Mantel test indicate that adjacent populations are in fact one, and furthermore confirms population clustering in 1, 3 and 4. Thus, the AMOVA results support the hypothesis that the way the meta-population is defined is much more responsible for genetic differentiation than the way that populations are defined. Less genetic differentiation was observed than expected for allopatric populations; a probable cause could be long distance dispersal via pollen or seed. This was observed in shared alleles from cluster 1 and cluster 4 even with a distance > 600 km between populations.

Measures of overall mean genetic diversity for \textit{H. cusickii} populations $U_H = 0.407$ were found to be very similar to the perennial \textit{Helianthus} species \textit{H. angustifolius} $U_H = 0.35$, \textit{H. verticillatus} $U_H = 0.48$, (Ellis et al. 2006) and \textit{Helianthus niveus} ssp. \textit{tephrodes} $U_H = 0.378$ (Mandel et al. 2013). The latter two mentioned are rare and endemic, while \textit{H. angustifolius} is widespread. Heterozygosity values of \textit{H. cusickii} were also comparable to $U_H = 0.42$, another endemic, perennial species with a restricted mountain desert habitat (Nybom 2004); and higher than the value $U_H = 0.219$ reported for the short-lived herbaceous perennial (Hamrick and Godt 1992). In comparison, \textit{H. annuus} cultivated inbred line were found to be higher than \textit{H. cusickii}, $U_H = 0.515$
(Tang and Knapp 2003). Actually, *H. cusickii* did not differ much in diversity from annual plants $UH_e = 0.46$ (Nybom 2004). Annual plant heterozygosities are typically higher due to their short-lived nature of rapid reproduction. Speculated hypotheses made for this research regarding genetic differentiation were correct, however not consistent with population structure findings.

**Growth Performance Study**

**Under Field Conditions**

Overall plants that were subject to field conditions, which included earlier planting and no irrigation displayed increased performance (survival and plant tissue growth). In natural environments, *H. cusickii* begins its vegetative growth from a perennial taproot in April, begins anthesis in May and completes seed set by July. Similarly, desert ephemeral plants have adaptations such as rapid phenological development for germination after first soaking of rain and phenotypic plasticity allowing for seed set with little rain (Mulroy and Rundle 1977; Turner and Begg 1981). While water resources are still available in early summer, environmental cues stimulate the plant’s genetic response to complete its life cycle and ensure reproductive success. The expansion of a deep root system in soil horizon layers permits water extraction and increased performance, while its fleshy taproot stores sugars under moisture limiting periods (Rauf 2008). However, a life strategy of early anthesis typically leads to a reduction in yields if soil water is depleted and the crop needs to rely on stored moisture in order to complete lifecycle (Turner and Begg 1981). This is due to the fact that perennial plants tend to be slow growing plants with a short temporal investment in
reproduction as compared with annual plants (Hirshfield and Tinkle 1975) and longer investment in the development of an underground storage system during stressful periods (Raunkiaer 1934; Walter and Schurr 2005). Although a daily watering regime allowed for increased plant tissue expansion, which in turn yielded larger plants, coupled with a change in soil type, these unfavorable abiotic factors resulted in low survival of plants. Over time, with selection pressure, this species may reach a new equilibrium between plant allocation to root structures and reproductive effort, and result in genotypes more favorable for domestic use (Dehaan et al. 2005).

If the annual and perennial life histories are recognized as merely quantitative traits that respond to selection, then minor changes in these traits have the ability to express either phenotype (Thomas et al. 2000; Dehaan et al. 2005). Van Kleunen et al. (2007) evaluated plastic responses expressed among internodal lengths when a perennial herb, *Ranunculus reptans* was exposed to periods of soil flooding. They concluded that the change in soil environment from flooding to non-flooding resulted in a decreased internode length, and an increase in overall fitness. Likewise *H. cusickii* plants had reduced shoot growth and flowering in field B where there was exposure to heavy periods of rain. Moreover, Kim and Donohue (2013) found plasticity in germination, plant size and morphological traits of a perennial herb, *Erysimum capitatum*. When they had grown this alpine species in a field environment, plants exhibited high mortality, but had a faster growth and reproduction of those that survived. Thus, induced abiotic changes can account for differential plant performance, which can be explained by a developmental
plasticity. This adaptive value found in plants is caused by a programmed development that can be modified to some degree (Maherali et al. 2009).

Leaf size and characteristics were found to vary most between populations, and can be explained by adaptation to varying microhabitats causing different ecotypes. Plants growing at low elevations had significantly larger leaf sizes (Appendix F) than those plants growing at higher elevations. There were observed variations in leaf characteristics with most plants exhibiting entire leaf margins that were lanceolate shaped, and a smaller portion of the plants exhibited undulated leaf margins and/or falcate (sickle) shaped. Undulated or falcate leaves reduce leaf area by creating vertical leaf orientation that contributes to water conservation in the plant. Another characteristic that decreases water evapotranspiration is the leaf pubescence noted in *H. cusickii*, which forms a boundary layer over leaf lamina preventing plant desiccation from wind and radiant heat energy. This was also observed when soil moisture was limited in plant species *Piriqueta caroliniana*; plant adaptations such as a reduction in leaf size and an increase in leaf pubescence density resulted in an increase in plant performance (Picotte et al. 2007).

*Helianthus cusickii* displayed a great difference in shoot growth as compared to *H. annuus* or *H. maximilliani* likely due to its programmed adaptation to harsh growing conditions. A reduced shoot growth allows for increased root growth, and further access to water in the soil column. High root shoot ratio contributed to allocation for root growth. In a nutrient rich environment such as agricultural conditions, growth is expected to be observed in plant vegetative structures rather than roots because it is
favored by selection (Cheplick 1995; Warembourg and Estelrich 2001; Dehaan et al. 2005). Consistent with hypothesis two, *H. cusickii* did exhibit significant variation in plant leaf characteristics, and important root traits, however these traits did not seem to have a direct relationship to adaptability under field conditions.

The use of genetic material from wild relatives remains an important source of useful variation for domestic crops particularly under environmentally stressful conditions. Advanced breeding methods (e.g., embryo rescue, somatic hybridization) are necessary for the introgression of genes from *H. cusickii* to *H. annuus* due to both pre and post-zygotic barriers. The fact that there were putative hybrids made does provide some evidence that crosses can yield seeds and selection was made possible. Conversely, if direct domestication of wild perennials is applied with the right selection pressures, perennial plants may have a chance to express a domesticated phenotype of many flowers contributing to higher yields, and large-scale genetic interventions might not be needed (Thomas et al. 2000; Dehaan et al. 2005). Without stressful environmental conditions, *H. cusickii* has the potential to adapt and present higher seed set under field conditions.

Populations 657 and 658 from Washington, populations 659, 661, 958 from Oregon, and population 958 from California had the greatest survival, vigor, and genetic diversity, although population 659 exhibited a lower genetic diversity. The genetic and phenotypic variation found within *H. cusickii* populations is a positive attribute for agricultural breeders and geneticists interested in the use of wild species for crop diversification and even more gives a level of confidence that the survival and vigor can be improved under simple selection. However, its current low survival rates under
agricultural field conditions suggestion that this species is a poor candidate for domestication and would be better suitable as a donor for genes in domesticated *H. annuus*. There is some preliminary evidence that *H. cusickii* can be crossed with domestic *H. annuus* and there may be variation for crossability, which can be selected for. Therefore in order for this species to be a useful source of perenniality or drought tolerance, several problems must be overcome including having plants be faster growing, more vigorous, larger in size and improving crossability between domestic *H. annuus*.

**Seed Dormancy Study**

Consistent with the evaluation of the growth performance of *Helianthus cusickii* for potential domestic use, the optimal conditions for breaking seed dormancy were examined. Both ethylene and gibberellic acid are natural occurring growth regulators within plants that promote cell elongation. The application of 2% aqueous gibberellic acid endogenously was found to stimulate a faster rate of germination for wild *H. cusickii* seed. Gibberellic acid breaks seed coat dormancy or the endosperm barrier, while stimulating germination (Finch-Savage and Leubner-Metzger 2006). A variety of environmental cues also break seed dormancy such as elevated temperature, water and light. During the springtime, temperatures naturally fluctuate between warm and cold. However, in this experiment, removing seeds from cold and subsequently exposing them to cycles of heat and cold slowed down the overall time to break seed dormancy. Seeds requiring a steady warmer temperature to germinate, rather than room temperature can explain this, and perhaps optimal temperatures were not reached.
There was a rapid increase of germination from the higher concentration of gibberellic acid, showing it to be the best growth regulator for wild sunflower seed germination. At low concentrations, both growth regulators were ineffective at breaking seed dormancy (not significantly different than the control treatment). Results were accordant with Kantar et al. (2013) in which higher concentrations of gibberellic acid were able to break *Helianthus tuberosus* tuber dormancy in less than 21 days. Hypothesis three which suggested ethylene would be a stronger hormone to break seed dormancy was not in agreement with findings.

Suggestions for Future Work

The goal of this experiment was to perform an initial evaluation of a wild, perennial sunflower for domestic use. Genetic and phenotypic information gathered here on *H. cusickii* populations lays a foundation for future genetic studies and directs researchers to specific populations that may be most promising for use in a crop improvement program. Current domesticated sunflowers do not have a high capacity for drought tolerance (Rauf 2008), and yields are reduced with water limitation (Dragovic and Maksimovic 1995; Rauf 2008). I observed large differences between the root structure and depth of domesticated sunflowers and *H. cusickii* growing in the same environment. However, I was not able to conduct physiological comparisons. Therefore, additional studies, which aim to evaluate *H. cusickii*’s water storage capacity, or a root structure analysis, which examines the physiological responses of plants when exposed to varying water regimes. Of equal importance is to perform a plant yield analysis under
varying environmental stress (e.g., drought, salinity) and evaluate gene expression. If specific, unique, and potentially useful anatomical or physiological adaptations are identified in *H. cusickii*, QTL analysis may be necessary to facilitate the introgression of these traits into the crop sunflower genetic background.
REFERENCES


Warembourg FR, Estelrich HD. 2001. Plant phenology and soil fertility effects on below-ground carbon allocation for an annual (Bromus madritensis) and a perennial (Bromus erectus) grass species. Soil Biology and Biochemistry, 33(10):1291-1303.


REFERENCES
Germplasm accession information table. Represents the 17 accessions used in this study with GRIN identification number, use of germplasm in experiments, geographic locations, and elevations.

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Precipitation data in Salina, KS. Data gathered from National Ocean and Atmospheric Administration’s (NOAA), National Weather Service.

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<th>Month</th>
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Log likelihood for each K. When K is approaching a true value, L(K) continues to rise and has the highest variance between runs.
Delta K method output. Calculated based upon second order rate of change of the likelihood (Delta K). Delta K shows a large peak at true K value.
APPENDIX E
Leaf length and width measurements in plot A and B.

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(continued)

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APPENDIX F
The most robust populations in Plot A and B.

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<tr>
<td>Head diameter &gt;10 mm</td>
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<tr>
<td>Plant height &gt; 50 cm</td>
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<tr>
<td>Flowers/ plant &gt; 15</td>
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</tr>
<tr>
<td>Size &gt; 75% cover in 15 x 15 cm quadrat</td>
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