BIOCHEMICAL AND GENETIC CHARACTERIZATION OF RUBBER PRODUCTION IN PRICKLY LETTUCE (*Lactuca serriola* L.)

By

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To the Faculty of Washington State University:

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BIOCHEMICAL AND GENETIC CHARACTERIZATION OF RUBBER PRODUCTION IN
PRICKLY LETTUCE (Lactuca serriola L.)

Abstract

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Natural rubber (cis-1,4-polyisoprene) is one of the most important plant derived bio-
polymers used in the manufacture of thousands of products. Plant originated rubber is unique in
that the physical properties such as tensile strength, heat and impact resistance have yet to be
reproduced by synthetic polyisoprene. While many plants are able to make rubber polymers, few
can synthesize high molecular weight molecules. Prickly lettuce (Lactuca serriola L.), a
common weed, is one of the few. The following studies were conducted to assess the diversity,
genetics, the chemical and physical properties as well as the microscopic elements related to
prickly lettuce rubber production. Rubber from a collection of eastern Washington prickly lettuce
biotypes revealed variation in rubber content and quality, although all biotypes synthesized high
molecular weight rubber. Two distinct biotypes were crossed and selfed to generate an F2
segregating population. Plant phenotypes and genetic segregation of markers (EST-SSRs) were
collected to discover marker-trait associations or quantitative trait loci (QTL). Four main QTL
were discovered corresponding to the following traits; rubber molecular weight, leaf perimeter
and leaf lobing, stem counts and growth habit, and herbivory. A single marker association was
observed with the trait rubber polymer dispersity. Rubber extraction methods were evaluated and found that chemical extraction of dried, ground whole plant material yielded little rubber of low quality. Direct rubber extraction from tapped latex was the best extraction method. Rubber polymers were confirmed as cis-1,4-polyisoprene by nuclear magnetic resonance spectroscopy with little to no trans-polyisoprene detected. Physical property analysis showed that prickly lettuce rubber has properties equal to or better than rubber derived from Brazilian rubber tree. Prickly lettuce is a potential source of natural rubber having the desired physical properties for end-use production. Production feasibility will rely on rubber trait selection and extraction optimization to increase yield. Prickly lettuce can also serve as a model species to understand rubber biosynthesis as it is highly amenable to genetic and biochemical experimentation. These studies have extended the foundation of understanding towards the biosynthesis and utilization of rubber in prickly lettuce.
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Dedication

This dissertation is dedicated to my wife Camille Bell, my three children Dexter, Evelyn and Miles who have been along on this adventure; guys it’s almost time to get a dog.

To my parents William and Kathleen Bell who laid the foundation of faith and work.
Chapter 1
Prickly Lettuce as an Alternative Source of Natural Rubber

Literature Review

Most rubber products used globally are petroleum-based. In 2011, 57.8% of total worldwide rubber production and consumption was synthetically made (International Rubber Study Group). Synthetic rubber (predominantly a styrene-butadiene polymer) lacks many desirable end use characteristics, therefore synthetic polymers cannot completely substitute natural rubber. Natural rubber products have tensile strength, resilience, elasticity, abrasion and impact resistance, efficient heat dispersion, and malleability at cold temperatures, all of which are essential attributes in high performance applications such as medical devices and airplane tires. Because synthetic-based products are derived from a non-renewable resource, natural rubber will be needed to supplement and eventually replace synthetic productions as oil supplies are exhausted. Domestication and commercialization of a rubber-producing crop is the best short and long-term solution to the future decline in synthetic rubber products, especially for developed countries such as the United States, the second largest consumer of natural rubber (Hayashi 2009).

Natural Rubber Production

Natural rubber (cis-1,4-polyisoprene) is an important plant derived bioproduct essential in the manufacture of over 40,000 products (Mooibroek and Cornish 2000). Brazilian rubber tree [Hevea brasiliensis (Willd. ex A. Juss.) Müll. Arg.], a tropically endemic species is the source of industrialized natural rubber production. Harvest of natural rubber has steadily increases since it was first discovered and commercially produced. Worldwide manufacture of natural rubber reached 10,978,000 t with consumption at 10,919,000 t in 2011. Yearly production is at demand
levels with minimal surpluses reported. In 2010, there was a 377,000 t deficit in consumption versus production (International Rubber Study Group). Natural rubber has strategic importance as that the current source of natural rubber is endemic to tropical regions. Security of supply chains for countries were the rubber tree cannot be cultivated is a high priority. The search continues for plant species that are able to efficiently generate the quantity and quality of natural rubber as their tropical counterparts.

Currently all natural rubber used in large scale production comes from a single species, the rubber tree. Native to the Amazon basin, the rubber tree has been propagated and cultivated in many tropical regions throughout the world, most notably the south Pacific region. Thailand, Indonesia and Malaysia are the top three leading world exporters of natural rubber (Hayashi 2009). Reliance on a single plant species has foreseeable disadvantages and potential dangers. The current crop of plantation-grown trees is almost entirely made up of cloned individuals. The lack of genetic variability renders entire plantations susceptible to pathogen attack as observed on cultivated trees in South America damaged by leaf blight (Davis 1997; Le Guen et al. 2004). As rubber trees can only be cultivated in tropical climates, countries in temperate regions are dependent upon the few producing countries for the entire supplies of natural rubber. Economic and political instabilities in many of these developing countries could jeopardize imports. In addition, supplies could diminish as growers switch to more valuable crops such as oil palm and rattan (Belcher, Rujehan, and Achdiawan 2004).

Furthermore, latex from the rubber tree causes latex allergies. Proteins present in rubber tree latex products, most notably hevein, induce allergic reactions in >7 % of the population (Rolland, Drew, and O’Hehir 2005). Health care workers and patients requiring repeated medical procedures or long-term hospitalization are at increased risk of developing latex hypersensitivity
(Lai et al. 1997; Tosi et al. 1993). The allergic response is an immunoglobulin E (IgE)-mediated Type 1 reaction which in serious cases leads to life-threatening anaphylaxis (Slater 1989).

Esau (1965) identified 12,500 plant species across twenty families and 900 genera that produce latex. Of these, rubber could be produced from the latex of 1800 of the species across eight families and 300 genera (Metcalfe 1967). While there have been attempts to identify various temperate or tropical plant species as a source of natural rubber, success has been limited (Buchanan et al. 1978; Carr and Bagby 1987; Hall and Goodspeed 1919; Mitchell, Rice, and Roderick 1942; Polhamus 1958). Very few plant species produce latex that can be used to produce durable high molecular weight rubber or simply cannot efficiently produce enough latex on a per hectare basis as the rubber tree (Mooibroek and Cornish 2000). Currently, only *Parthenium argentatum* Gray., commonly known as guayule, is used for commercial production of hypoallergenic latex products and only on a small scale (Cornish 2001). Production of rubber products from guayule may meet the demands of medical markets; however, guayule will not produce sufficient amounts to supply larger markets such as tire production (Cornish 2001). Guayule does not tolerate cold winters experienced by much of the temperate regions of the world and more importantly it cannot be harvested as an annual crop as rubber is produced in bark parenchyma cells. One season's growth yields little bark parenchyma and rubber. Additional rubber-producing crops that are fast growing and harvested annually are needed to fill the larger non-medical market.

Russian dandelion (*Taraxacum kok-saghyz* L.E. Rodin.) is a temperate annual from Kazakhstan identified in the 1930s as a producer of latex (Ulmann 1951). In terms of research resources spent, Russian dandelion closely follows guayule and has promise as an alternative rubber source. Rubber from this plant was used to make various products during WWII when
supply lines to the tropics were cut off (Polhamus 1962; Whaley and Bowen 1947). Latex is produced in the tap root and has molecular weight rubber similar to or greater than that of the rubber tree. For rubber production to be economical, yields must be increased and biorefinery methods employed where all plant components are utilized (Van Beilen and Poirier 2007).

**Prickly Lettuce Rubber Production**

The best rubber-producing crop for temperate regions will be a fast growing annual that produces large amounts of biomass. Annual crops are more easily incorporated into crop rotation systems and can be planted or removed in response to market fluctuations and independent grower requirements (Van Beilen and Poirier 2007). Perennial plant species such as trees or shrubs often have higher starting costs, have a lag from input to harvest and remain in place for several growing seasons, making them less suited to changing market demands. Preferably, the rubber producing species will also be tolerant and well adapted to cultivation on marginal land, alleviating competition with food crops, as seen in corn-ethanol production (Babcock and Fabiosa 2011). The required tolerance and adaptability traits are often present in weedy species. Weeds are well adapted to the region they infest, require low production inputs, have high water-use efficiency, and are largely resistant to disease and insect pests. Many of these species have high photosynthetic efficiency and consequently high biomass yields or are drought resistant. In some cases, populations of herbicide resistant weeds may provide production options for their development as a crop. Weedy plants could provide value-added bioproducts that increase the economic feasibility of establishing them as crops for biomass and bioproducts. Prickly lettuce (*Lactuca serriola* L.) is one such weed, and has been identified as a plant species able to produce high molecular weight rubber (Bushman et al. 2006).
Interestingly, the genus *Lactuca*, of which prickly lettuce is a prominent member, has long been recognized as a potential source of rubber (Bushman et al. 2006). In 1913, a report detailed the analysis of latex from two lettuce species, *Lactuca canadensis* L. and *L. scariola* L. (Fox 1913). Both species secreted latex from all areas of the plant. Rubber content of the latex was measured at 2.2 % in *L. canadensis* and 1.6 % in *L. scariola*. The variability of rubber content and the ability to produce rubber among lettuce genotypes was also described by the Clemson Agriculture Center (Mitchell, Rice, and Roderick 1942) and the US Department of Agriculture (Polhamus 1958). The reports described differences in latex rubber content of several lettuce species with negligible differences in percent rubber extracted from whole plants. None of these reports discussed the purity or composition of the latex, the molecular weights of the rubber in the latex, or the condition of the tissues previous to extraction – that work was completed by Bushman et al. in 2006.

Bushman et al. (2006) examined prickly lettuce, accession UG92G488, and a cultivated lettuce cultivar (*Lactuca sativa* L. cv. Salinas) accession UC96US23 to determine characteristics of natural rubber in those two lettuce species. They demonstrated that both species produce latex containing desirable high molecular weight (>1 million g mol \(^{-1}\)) *cis*-1,4-polyisoprene with very narrow dispersity that should lead to excellent product characteristics after vulcanization. Bushman et al. (2006) also concluded that genetic, developmental, or environmental influences could impact latex molecular weight and suggested optimizing the genetic, physiological, and environmental conditions for the synthesis of higher molecular weight rubber for prickly lettuce. In order to consider the potential prickly lettuce has as an alternative source for latex and rubber bioproducts, continued research and development must be conducted.

**Botanical Characteristics of Prickly Lettuce**
Prickly lettuce, a native of Europe, is a winter/spring annual that grows as a basal rosette during the vegetative phase. Upon bolt initiation, one or more stems grow to 2 m in height. A deep tap-root with external branching 5 cm below the soil surface facilitates water uptake under drought conditions (Gallardo et al. 1996; Jackson 1995). Leaves are alternate and clasping with bristles on the undersurface along the mid-vein and are irregularly-pinnately toothed (Weaver and Downs 2003). Under certain environmental condition, prickly lettuce has the ability to orient cauline leaves in a north-south direction perpendicular to the soil surface coining the designation “compass plant”. Modified leaf orientation affects the diurnal pattern of solar irradiance in turn affecting leaf temperature and total transpirational water loss enhancing photosynthetic output and reproductive ability late in the growing season (Werk and Ehleringer 1984, 1986). The stems contain a milky sap, hence the generic name- *lacta*, for "milk," referring to the milky latex sap in stem and leaf tissue. Bolting is facilitated by a long-day photoperiod, however, multiple partially dominant early flower (Ef) genes (Ryder and Milligan 2005) and a flowering locus (FT) gene (Fukuda et al. 2011) derived from cultivated lettuce experiments contribute to a wide range of bolting phenotypes. Flowers are mainly self pollinated yet out-crossing events occur and are dependent on climatic conditions and pollinator abundance (Watts 1958; D’Andrea, Felber, and Guadagnuolo 2008). Mature pappus bearing achenes are primarily wind disseminated and remain viable in the seed bank from 1 to 3 years (Marks and Prince 1981; 1982; Alcocer-Ruthling, Thill, and Shafii 1992). Maximum reproductive capacity is predicted to be 200,000 seeds per plant (Weaver and Downs 2003) depending on growth conditions. Prickly lettuce is an abundant and widespread weed in meadows, woodlands, stream channels, and waste places throughout most of the United States, even in areas with very low rainfall, blooming from May to September (Weaver and Downs 2003). Prickly lettuce has become one of the most common and
troublesome weeds in the inland Pacific Northwest (Burke et al. 2009). It is well suited to the dryland areas of eastern Washington and is capable of producing abundant above ground biomass, although its growth characteristics haven’t been fully studied.

**Herbicide Resistance in Prickly Lettuce**

In 1987, a sulfonylurea herbicide-resistant prickly lettuce biotype was identified in a no-till, continuous winter wheat field that had been treated with sulfonylurea herbicides for 5 years (Mallory-Smith, Thill, and Dial 1990). Sulfonylurea herbicides inhibit acetolactate synthase (ALS), a key enzyme in the production of the branched-chain amino acids. That resistance was controlled by a single nuclear gene with incomplete dominance (Mallory-Smith et al. 1990). Since that discovery in Idaho, four other biotypes of ALS–resistant prickly lettuce have been reported (Heap 2012). In preliminary research, more than 55% of sampled biotypes from eastern Washington were found resistant to ALS herbicides (Stevens and Burke 2009). More recently, 2,4-D resistance was confirmed in eastern Washington (Burke et al. 2009). Prickly lettuce 2,4-D resistance appears to be controlled by a single codominant gene with resistant biotypes showing reduced 2,4-D absorption and translocation compared to susceptible biotypes (Riar et al. 2011). These traits would readily lend themselves to cultivation of prickly lettuce and closely related cultivated lettuce.

**Agronomic and Climatic Requirements of Prickly Lettuce**

Prickly lettuce is a native to a summer-dry Mediterranean climate (Gallardo et al. 1996) and is considered a drought-tolerant species (Werk and Ehleringer 1985; 1986). Prickly lettuce can be found in many different communities that have some disturbance, particularly crops grown with reduced tillage (Reed 1970; Schoennagel and Waller 1999). As a native of a Mediterranean climate and a ruderal, prickly lettuce is adapted to the production practices of the
inland Pacific Northwest and is particularly adapted to the dryland production regions of eastern Washington. Germination, which is largely controlled by temperature and the quality of light (Marks and Prince 1979), occurs soon after the seeds are shed. Prickly lettuce seed is readily viable when it is shed. However, dormancy can be induced by allowing the seeds to imbibe prior to withholding water. The main period of germination in Washington appears to be in early spring, although germination can occur in late autumn and early winter and can continue well into summer as conditions permit. Thus, the life cycle in a single colony can range from winter annual to summer annual (Marks and Prince 1981).

Prince et al. (1978) noted that prickly lettuce is found primarily between latitudes 30 and 55 in the northern hemisphere. Rousseau (1968) observed that the distribution of prickly lettuce in Quebec was limited to areas with more than 3000 degree days. Although prickly lettuce is thought to prefer dry soils (Reed 1970; Hanf 1983), it tolerates a wide range of soil types. Prickly lettuce can be found growing on anything from rocky outcrops to fertile rich loams. Although no production information is available for prickly lettuce, it is reasonable to expect that if production was attempted, many of the practices currently used in cultivated lettuce would be applicable.

**Lettuce Genetic Resources**

Prickly lettuce is closely related to, and most likely the progenitor of cultivated lettuce. It is believed the two species are conspecific (Koopman et al. 2001) and are easily crossed to produce viable offspring. Cultivated lettuce is grown throughout the world and consumed for its nutritious foliage (Mou 2012; Nicolle et al. 2004). Cultivated lettuce has 9 chromosomes and a genome size of approximately 2600 Mb. Prickly lettuce also has 9 chromosomes and presumably a similar genome size. The Compositae Genome Project (CGPDB;
http://compgenomics.ucdavis.edu) is the most comprehensive genetic resource for study of the Compositae family including cultivated lettuce and four closely related lettuce species, L. serriola L., L. saligna L., L. virosa L. and L. perennis L., ordered in proposed phylogenetic relatedness to cultivated lettuce. The genetic and genomic architecture of cultivated lettuce and its relatives has been well documented and includes a large inventory of molecular markers. Variable marker types include RFLPs (Kesseli et al. 1991; 1994), RAPDs (Waycott and Fort 1994), ISSRs (Vicente et al. 2008), genomic/EST-SSRs (Riar et al. 2011; Simko 2009; Van de Wiel et al. 2010), AFLPs (Jansen et al. 2005; Jeuken et al. 2001; Koopman et al. 2001), TRAPs (Hu et al. 2005), SNPs (Moreno-Vázquez et al. 2003) and SFPs (Van Leeuwen et al. 2009). The developed molecular markers have been effectively utilized for linkage mapping (Truco et al. 2007), gene-tagging, QTL mapping (Jeuken et al. 2001) and genetic diversity studies (Kuang et al. 2008; Riar et al. 2011). Although cultivated lettuce is the main research focus, many of the molecular markers are transferable and have been used between lettuce species. Most recently the cultivated lettuce genome has been released (www.lgr.genomecenter.ucdavis.edu/) and will become a valuable asset in cultivated lettuce research as well as close lettuce relatives through syntenic genetic comparisons.

Prickly lettuce is important in cultivated lettuce breeding as donors of resistance genes (Lebeda 1998; Lebeda et al. 2002; Reinink 1999; Ryder 1999; Sicard et al. 1999; Zohary 1991). On the basis of the gene-pool concept (Harlan and Wet 1971), Lactuca species were categorized into three gene pools based on their relationship to cultivated lettuce (McGuire et al. 1993). The primary gene pool is represented by numerous cultivars of cultivated lettuce, primitive landraces, and wild species where crossing barriers do not exist. Prickly lettuce is the primary
representative as the progenitor species of this gene pool (de Vries 1997) and is a primary beneficiary of the resources devoted to the development of cultivated lettuce genetic resources.

**Rubber History**

The first documented uses of plant derived rubber were made by ancient Mesoamerican peoples as early as the 1600s B.C. (Hosler et al. 1999). The latex from the Panama rubber tree (*Castilla elastica* Sesse.) was mixed with juice from the morning glory vine (*Ipomoea alba* L.) producing a coagulated rubber material which could be molded into the desired shape (Hosler et al. 1999). The Mesoamerican name for this material is *cauchu* meaning “weeping wood”. The invention of coagulated rubber gave rise to its application as a material to make human figurines, and in tool preparation by wrapping handles or hafting stone heads to wooden handles. Aztecs and Mayans used rubber to waterproof clothing (Evans 2008), while Mexicans used it to cover feet in a waterproof form of a primitive shoe (Wolf and Wolf 1936). Sixteenth century Spanish conquistadors observed “the ball game”, the first team sport complete with court, spectator stands and solid rubber ball (Herrera y Tordesillas et al. 1725). In addition to religious significance, ball game contenders also gambled for land, slaves, and valuables (Hosler et al. 1999).

Although several European observers saw rubber as a curious and interesting material, two hundred years passed before any mention of rubber uses were suggested. Samples of India rubber were used in France as erasers in 1752 (Porritt 1927). British intellectual Joseph Priestly is often referenced as the inventor of the eraser by citing a 1770 note that he had “seen a substance excellently adapted to the purpose of wiping from paper the marks of a black lead pencil” (Priestley 1770) however Priestly never claimed the discovery. Another seventy years would lapse before real manufacturing efforts of rubber-derived goods was undertaken.
By 1823 Charles Mackintosh of England began making double-textured rubberized cloth by spreading naptha-dissolved rubber solutions over fabric with the intention of waterproofing the material (Levitt 1986). His raincoats forever became known as ‘mackintoshes’. At the same time Thomas Hancock developed rubber manufacturing machinery such as the mastication mill and calendar sheet roller. His factory also produced rubberized fabrics as well as rubber hoses, bumpers, carriage tires and other goods (Hancock 1857). Manufacturing was at full swing yet products were met with dissatisfaction because of their sensitivity to temperature extremes and horrible smell. At low temperatures the rubber became hard and brittle while in hot weather, sticky and soft. A processing invention discovered in 1839 would alleviate these flaws and revolutionize the rubber goods industry.

Vulcanization, named after the ancient Roman god of fire Vulcan, would be the saving grace of rubber manufacturing. The American Charles Goodyear is credited with the invention, a process of heating rubber with sulfur. Much of Goodyear’s career was met with failure and misfortune, yet he had invested himself in solving the problems associated with rubber properties. Many of his experiments were conducted in a make-shift home laboratory where he experimented with numerous rubber additives (Anon. 1958). In 1838 he met rubber factory owner Nathaniel Hayward who was using sulfur as a rubber drying agent. Goodyear tried sulfur as an additive and found that after heating at high temperature, some of the rubber charred but most had cured (New Haven Colony Historical Society 1975). Vulcanization of rubber created a chemical resistance, temperature and pressure resilient material that had elastic properties never before encountered. Upon further perfection of the process, Goodyear took out a patent on the method in 1844 (Goodyear 1844).

**Rubber Properties**
Rubber is an elastomer that possesses unique chemical and physical properties. Polyisoprene in its original state is elastic, waterproof, and moldable. Yet native polyisoprene has limited use capabilities. At low ambient temperatures it becomes brittle, while at high temperatures tackiness and increased malleability becomes problematic. The basis for these properties is independent movement of the isoprene polymers in relation to one another. Polymer chain entanglements are the only interacting junctures to hold shape (Mark and Erman 1994). It was not until the discovery of vulcanization that the true potential of rubber was realized. Vulcanization introduces cross-linkages of the isoprene polymers mainly through sulfur bridges. Sulfur cross-linking is achieved when rubber dienes react with sulfur in the presence a catalyst, under heat and pressure. Numerous vulcanization methods have been developed depending on the end-use requirements of the material. The most important vulcanization techniques are press, open, continuous, and cold vulcanization (Nagdi 1993). Vulcanization increases the retractile force of distorted polymer chains when under mechanical stress and reduces the amount of permanent deformity remaining when deforming force is removed (Mark and Erman 1994). Prolonged vulcanization with high sulfur produces a very hard, durable material known as ebonite or vulcanite (Kemp and Malm 1935).

Once vulcanized, rubber exhibits exceptional viscoelastic properties. The theory of rubber elasticity states that the retractile force to resist deformation is proportional to the number of network-supporting polymer chains per unit volume of elastomer (Flory 1953; Mark and Erman 1994). In short, the amount and type of cross-links imparts the physical properties of the polymer in its changed state. There is a correlation between plasticity and elasticity that is directly related to the amount cross-linking. Hysteresis, a ratio measure of this relationship, decreases as cross-link density increases. Rubber polymers become more plastic-like and hard
with high cross-linking. Optimal cross-link densities maximize tear strength, fatigue life, toughness and tensile strength. Vulcanization beyond the optimal region diminishes these properties (Mark and Erman 1994).

To make high-quality, high performance vulcanized rubber products the natural rubber starting material must meet certain criteria. Polymer chain length or molecular weight and molecular weight distribution (dispersity) have a direct relationship to the processability of the rubber (Swanson, Buchanan, and Otey 1979). In general, longer polymer chains with narrow D, give the best end-use performance.

**Anatomy and Biochemistry of Polyisoprene Synthesis**

Rubber consists of long chains of isoprene configured in a cis-1,4 orientation, otherwise known as polyisoprene. Natural rubber refers to the coagulated or precipitated polyisoprene isolated from the latex of rubber producing organisms. In plants, isoprene is synthesized via two independent and non-homologous cellular pathways. In the cytosol the well characterized mevalonate pathway (MVA) prevails. This pathway was first described in the 1950s and, for a time, was believed to be the only means of isoprene biosynthesis (McGarvey and Croteau 1995) although it was not present in most bacteria (Zhou and White 1991). More recently another plastidal pathway was discovered and described as the non-mevalonate, 2C-methyl-D-erythritol-4-phosphate or 1-deoxy-D-xylulose-5-phosphate pathway (MEP/DOXP) (Rohmer et al. 1993). The products generated from both pathways are isopentyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), the precursors for all isoprenoid derived compounds. Brazilian rubber tree rubber biosynthesis indicates MVA pathway as the source of isoprene subunits while isoprene from the MEP/DOXP is incorporated into diterpenoids and carotenoids (Sando et al. 2008).
Prickly lettuce has the same or similar anatomical features as other rubber producing plants. Rubber is made and stored in specialized vascular cells called laticifers. Laticifer producing plants have either articulated laticifers where cells are connected via anastomoses, as with prickly lettuce, or are non-articulated (Kekwick 2001). Guayule does not possess laticifers but rather produces latex in specialized bark parenchyma cells (Ross 1908). Within the cytoplasm of laticifers cells are found spherically shaped, organelle-like, rubber particles. Rubber particles are surrounded by a fatty-acid mono layer membrane imbedded with the enzymatic machinery necessary for isoprene transfer and polymer elongation within the hydrophobic core of the particle (Cornish 2001). The protein and lipid components found in laticifer membranes are species specific. Within species, the lipid membranes are composed of the same lipid components of the cells from which they are derived suggesting the absence of a highly conserved structural and chemical composition of particle membranes (Cornish 2001). Particle associated proteins between species are also highly variable, differing in overall quantities and structure of the polymer producing enzyme(s). Rubber tree rubber is the most protein complex to date with over 80 particle associated proteins (Cornish 2001), while guayule has less than ten (Siler et al. 1997).

Multiple studies have implicated several potentially important enzymes that are necessary for isoprene polymerization from various species. Because particle associated proteins do not have solubilized activity, conclusive evidence of specific rubber transferase protein(s) remains elusive. Current studies rely on isolation of enzymatically active washed rubber particles (WRPs). In all cases polymer elongation requires a short trans-allylic diphosphate primer molecule such as farnesyl pyrophosphate (FPP), geranyl pyrophosphate (GPP), or geranyl–geranyl pyrophosphate (GGPP) to serve as initiators upon which monomers of IPP are
sequentially added (Archer and Audley 1987; Cornish 1993; Puskas et al. 2006). FPP is the substrate of choice for in vivo polymerization (da Costa, Keasling, and Cornish 2005; Espy et al. 2006). Divalent metal ion cofactors such as Mg$^{2+}$ or Mn$^{2+}$ are also requisite for enzymatic activity (Scott et al. 2003). Rubber cis-polyisoprene transferase enzyme (EC 2.5.1.20), a rubber particle membrane-bound cis-prenyltransferase (CPT) is thought to be able to switch newly condensed IPP units from trans to cis configuration during polymer elongation (Tanaka 1989).

Rubber tree CPT identity and characterized function has been reported several times (Archer and Cockbain 1969; Cornish 1993; Light and Dennis 1989). Similar CPT activities have been identified in other rubber bearing plants such as Ficus elastica Roxb. ex Hornem. and Ficus carica L. (Cornish and Siler 1996; Kang et al. 2000), guayule (Madhavan and Benedict 1984), Russian dandelion (Schmidt et al. 2010) and lettuce (Bushman et al. 2006). Other particle membrane associated proteins implicated in cis-polyisoprene biosynthesis including rubber elongation factor (REF) (Dennis et al. 1989; Dennis and Light 1989) and small rubber particle protein (SRPP) (Kim et al. 2004; Oh et al. 1999; Schmidt et al. 2009). Interestingly, immunological studies from three rubber producing species, Ficus carica L., Ficus benghalensis L., and Brazilian rubber tree, found that CPT was not localized on particle surfaces of any of the three studied species. Rubber tree SRPP was found on the rubber tree particles but was not found on either F. carica, or F. benghalensis particles (Singh et al. 2003) suggesting that CPT is not part of the rubber transferase complex and SRPP is not required for polymer elongation. Further studies of the protein(s) or protein complex responsible for polymer elongation and chain length determination are needed. Because prickly lettuce is one of the few latex bearing species that produces long chain polyisoprene, it is an excellent model species for further elucidation of high molecular weight rubber biosynthesis.
Rubber Extraction Methods

To utilize plant synthesized rubber, intact particles or freed polymers must be tapped or extracted from the plant. In larger woody tree species such as Brazilian rubber tree, tapping is the method of choice. Tapping of rubber tree latex is usually done once every 2-3 days for 9 months of the year. Collection is made by cutting a thin layer of bark just above the cambial layer and collecting drips of latex into a cup. Anti-coagulating agents, usually ammonia, are added during collection to preserve the latex in liquid form or formic acid is added to the latex to coagulate the suspended rubber particles and collected as cup lump rubber (Sakdapipanich and Rojruthai 2012). Other rubber producing species under commercial investigation are not amenable to tapping procedures therefore more destructive methods must be used to release then isolate rubber particles or polymers from plant tissue. Plant processing in this manner presents challenges in maintaining rubber integrity throughout the extraction process. Processing of bulk rubber from guayule has been challenging due to low bulk viscosity and entrained co-extracted resin causing oxidative and thermal polymer degradation (Schloman 2005). The chemical and physical properties of rubber isolated from plant tissue is an artifact of the polymer structure within the latex-bearing cells and the method of polymer extraction as noted in guayule extractions (Schloman 2005).

In general there are two main methods of rubber extraction, solvent extraction and floatation. Chemical extraction of rubber involves isolation of rubber polymers from plant material and other metabolites using selective solvents after grinding of plant material. Solvents can be added sequentially or simultaneously. In sequential solvent extraction a polar organic solvent such as acetone is first used to extract pigments, polar lipids, resins, and other polar metabolites while leaving non-polar hydrocarbons. A second, non-polar dissolving solvent such
as hexane, cyclohexane, toluene or benzene is used to then solubilize rubber. All other non-polar compounds such as waxes and lipids could be present as impurities in the rubber fraction. Simultaneous solvent extraction includes addition of a single solvent such as xylene (Wagner and Parma 1988) or an azeotropic mixed solvent system (Beinor and Cole 1986). Simultaneous extraction produces a mixture of dissolved resin and rubber. Precipitation of the rubber by addition of acetone or alcohol is a means of further purifying the rubber polymers. For most small scale analytical purposes, chemical extraction has been the method of choice. Researchers have used various chemical extraction methods to analyze rubber from diverse plant derived sources (De Rodriguez and Kuruvadi 1991; Hammond and Polhamus 1965; Mekkriengkrai et al. 2004; Nurthen et al. 1986; Pearson et al. 2010; Pearson, Cornish, and Rath 2013; Spanò et al. 2012). However, on a large scale, chemical extraction is not the method of choice due to costs associated with solvent purchase and recovery and flammability hazards. Rubber from guayule has been extracted for bulk rubber production through chemical extraction methods (Hamerstrand and Montgomery 1984; Schloman 2005).

Floatation extraction methods have also been used in lab scale experiments and bulk rubber production. This method usually involves milling and centrifugation steps in an aqueous buffer, the addition of acid to coagulate rubber or creaming agents to float the rubber particles is a common practice (Buranov and Elmuradov 2010; Cornish and Backhaus 1990). Direct water milling and floatation methods have been developed for application to fleshy plants (Eskew and Edwards 1946; Stamberger, Koenig, and Hanslick 1946).

The efficient extraction of rubber from a highly complex plant matrix continues to be a problem in all facets of natural rubber production. Brazilian rubber tree tapping of latex bypasses impurities introduced from whole plant extraction, however tree tapping and latex collection is
performed by hand, decreasing efficiency, and latex stability must be maintained by addition of anti-coagulants and anti-oxidants. Plants that must be destroyed to harvest rubber must have optimized extraction procedures that increase rubber yield and decrease rubber degradation. Efficient, non-degradative extraction of rubber from prickly lettuce will be a major hurdle to overcome to produce high quality bulk rubber.

**Research Objectives**

The objective of this dissertation was to study the rubber making potential of a prevalent, well adapted, troublesome weed in eastern Washington. The goal was to characterize rubber production between various prickly lettuce biotypes and further identify molecular markers that would be useful in development of this weed as a crop plant. The findings of this dissertation are divided into five chapters that summarize: 1) Rubber content and quality screen of eastern Washington prickly lettuce biotypes; 2) Genetic map development and marker association with rubber producing traits; 3) Rubber extraction methods and physical properties of rubber extracted from prickly lettuce biotypes; and 4) Microscopic description of prickly lettuce rubber particles; and 5) Future direction from this research. Additionally, a chapter on science and philosophy as seen by this scientist concludes the chapter sections.
Literature Cited


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Chapter 2
Rubber Content and Quality Screen of Twenty Eastern Washington Prickly Lettuce Biotypes

Abstract

To explore the potential of prickly lettuce (*Lactuca serriola* L.) as an alternative source of natural rubber, twenty eastern Washington biotypes were collected and grown in common greenhouse and field gardens. The biotypes were morphological variable and many were Group 2 herbicide resistant. Prickly lettuce latex was tapped from stems of both greenhouse and field grown plants. Collected latex was dried under vacuum for 48 h at 35 ºC and solvent extracted to yield percent latex components by weight. ANOVA indicated that latex composition was similar among environments. Prickly lettuce latex composition, averaged over environment, was composed of water (61.7 %), insoluble (23.2 %), acetone soluble (13.8 %), and hexane soluble material (3.2 %). The amount of extractable rubber material ranged from 2.2 % to 4.9 % by weight between biotypes. The rubber fraction was further analyzed by gel permeation chromatography (GPC) HPLC, with refractive index detector to evaluate rubber (*cis*-1,4-polyisoprene) polymer chain length. GPC software was used in conjunction with polystyrene standards to give an estimation of average molecular weight (Mn), weighted average molecular weight (Mw), and D. All collected biotypes have an average polymer Mw considered high quality (>1x10^6 g mol⁻¹) with several producing polymers greater than 2x10^6 g mol⁻¹. Average polymer D was 2.91 and ranged from 2.31 to 3.99 between biotypes. Identification of prickly lettuce biotypes with favorable rubber producing traits will support further studies on rubber synthesis control mechanisms and assist in selection of rubber lettuce cultivars.
Introduction

Weeds are potential sources of plant derived bio-products and biomass. Weeds are well adapted to grow with little water and nutrients, and are usually resistant to disease and insect pests. Many weed species have high biomass yields due to photosynthetic efficiency. The use of herbicides and their inherent selection pressure has contributed to the development of herbicide resistant weed populations, a trait that could be used in their development as a crop. Weedy plants could provide value-added bioproducts that increase the economic feasibility of establishing them as crops for biomass and bioproducts. One such weed, prickly lettuce (*Lactuca serriola* L.), has been long been recognized as a plant species able to produce high molecular weight rubber (Bushman et al. 2006), an important plant bioproduct.

Demand for natural rubber continues to rise. Since natural rubber was first discovered and commercialized, production has steadily increased (International Rubber Study Group 2012). Natural rubber is used to manufacture over 40,000 products including 400 medical devices (Mooibroek and Cornish 2000) and is an important constituent of 600 components of a single passenger car (Dick 2003). In 2011, worldwide manufacture of natural rubber reached 10,978,000 t with consumption at 10,919,000 t. Yearly production is at demand levels with minimal surpluses reported. In 2010, there was a 377,000 t deficit in consumption versus production (International Rubber Study Group 2012). Natural rubber has strategic importance given that the single current source of natural rubber for large scale production, the Brazilian rubber tree [*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.], is endemic to tropical regions. Security of supply chains to countries were rubber cannot be produced is a high priority. The Brazilian rubber tree is native to the Amazon basin yet has been propagated and cultivated in many tropical regions throughout the world, most notably the south Pacific. Thailand, Indonesia
and Malaysia are the top three leading world exporters of natural rubber (Hayashi 2009).

Reliance on a single plant species has foreseeable drawbacks and real potential dangers. The current crop of plantation-grown trees is almost entirely made up of cloned individuals. The lack of genetic variability renders entire plantations susceptible to pathogen attack as observed on cultivated trees in South America, damaged by leaf blight (Davis 1997; Le Guen et al. 2004). As rubber trees can only be cultivated in tropical climates, countries in temperate regions are dependent upon those few producing countries for the entire supplies of natural rubber. Economic and political instabilities in many of these developing countries could jeopardize imports. In addition, supplies could diminish as growers switch to more valuable crops such as oil palm and rattan (Belcher, Rujehan, and Achdiawan 2004). Therefore alternative plant species that are able to efficiently generate the quantity and quality of natural rubber as their tropical counterparts are of high demand.

Additionally, latex from the rubber tree causes latex allergies. Proteins present in rubber tree latex products, most notably hevein, induce allergic reactions in > 7% of the population (Rolland, Drew, and O’Hehir 2005). Health care workers and patients requiring repeated medical procedures or long-term hospitalization are at increased risk of developing latex hypersensitivity (Tosi et al. 1993; Lai et al. 1997). The allergic response is an immunoglobulin E (IgE)-mediated Type 1 reaction which in serious cases leads to life-threatening anaphylaxis.

Most rubber products used globally are petroleum-based. In 2011, 57.8% of total worldwide rubber production and consumption was synthetically made (International Rubber Study Group 2012). Synthetic rubber (predominantly a styrene-butadiene polymer) has not entirely replaced natural rubber because synthetic manufactures have yet to reproduce the desirable characteristics present in natural rubber. Natural rubber products have high tensile
strength, resilience, elasticity, abrasion and impact resistance, efficient heat dispersion, and malleability at cold temperatures, all of which are essential attributes in high performance applications. As synthetic-based products are derived from a non-renewable resource, natural rubber will be needed to supplement and eventually replace synthetic productions as oil supplies are exhausted. Domestication and commercialization of rubber-producing crops is the best short and long-term solution to the future decline in synthetic rubber supply, especially for developed countries such as the United States, the second largest consumer of natural rubber (Hayashi 2009). Esau (1965) identified 12,500 plant species across twenty families and 900 genera that produce latex. Of these, rubber could be produced from the latex of 1800 of the species across eight families and 300 genera (Metcalfe 1967). While there have been attempts to identify various temperate or tropical plant species as a source of natural rubber, success has been limited (Bowers 1990; Buchanan et al. 1978; Carr and Bagby 1987; Hall and Goodspeed 1919; Mitchell, Rice, and Roderick 1942; Polhamus 1958). Very few plant species can produce latex for use in the production of durable high molecular weight rubber or simply cannot efficiently produce enough latex per hectare as the rubber tree (Mooibroek and Cornish 2000). Currently, only Parthenium argentatum (Gray), commonly known as guayule, is used for commercial production of hypoallergenic latex products and only on a small scale (Cornish 2001), and there is considerable interest in Russian dandelion (Taraxacum koksaghyz L.E. Rodin) (Van Beilen and Poirier 2007). Production of rubber products from guayule may meet the demands of medical markets. However, guayule will not produce sufficient amounts to supply larger markets such as tire production (Cornish 2001). Guayule does not tolerate cold winters experienced by much of the temperate regions of the world and more importantly it cannot be harvested as an annual crop as rubber is produced in bark parenchyma cells. One season of growth yields little bark
parenchyma and rubber. Russian dandelion is a temperate annual from Kazakhstan identified in the 1930s as a producer of latex (Ulmann 1951). In terms of research resources spent, Russian dandelion closely follows guayule and has promise as an alternative rubber source. Rubber from this plant was used to make various products during WWII when supply lines to the tropics were cut off (Polhamus 1962; Whaley and Bowen 1947). Latex is produced in the tap root and has molecular weight rubber similar to or greater than that of the Brazilian rubber tree. For rubber production to be economical, yields must be increased and biorefinery methods employed where all plant components are utilized (Van Beilen and Poirier 2007). Additional rubber producing crops that are fast growing and harvested annually are needed to fill the larger non-medical market.

The ideal rubber producing crop for temperate regions will be a fast growing annual that produces large amounts of biomass. Annual crops are more easily incorporated into crop rotation systems and can be planted or removed in response to market fluctuations and independent grower requirements. Biennial and perennial plant species such as trees or shrubs often have higher starting costs, and have a lag from input to harvest and remain in place for several growing seasons, making them less suited to changing market demands. Preferably, the rubber producing species will also be tolerant and well adapted to cultivation on marginal land so as to alleviate competition with food crops, as seen in corn-ethanol production (Babcock and Fabiosa 2011). The attributes described for the ideal temperate rubber producing plant are present in prickly lettuce.

The genus *Lactuca*, of which prickly lettuce is a prominent member, has long been recognized as a potential source of rubber (Bushman et al. 2006). In 1913, a report detailed the analysis of latex from two lettuce species, *Lactuca canadensis* L. and *L. scariola* L. (Fox 1913).
Both species secreted latex from all areas of the plant. Rubber content of the latex was measured at 2.2% in *L. canadensis* and 1.6% in *L. scariola*. The variability of rubber content and the ability to produce rubber among lettuce genotypes was also described by the Clemson Agriculture Center (Mitchell, Rice, and Roderick 1942) and the US Department of Agriculture (Polhamus 1958). The reports described differences in latex rubber content of several lettuce species with negligible differences in percent rubber extracted from whole plants. None of these reports discussed the purity or composition of the latex, the molecular weights of the rubber in the latex, or the condition of the tissues previous to extraction – that work was completed by Bushman et al. in 2006.

Bushman et al. (2006) examined prickly lettuce, accession UG92G488, and cultivated lettuce (*L. sativa* L. cv. Salinas) accession UC96US23 to determine characteristics of natural rubber in those two lettuce species. They demonstrated that both species produce latex containing desirable high molecular weight (>1x10^6 g mol^-1) cis-1,4-polyisoprene with very narrow Đ that should lead to excellent product characteristics after vulcanization. Bushman et al. (2006) also concluded that genetic, developmental, or environmental influences could impact latex molecular weight and suggested optimizing the genetic, physiological, and environmental conditions for the synthesis of higher molecular weight rubber for prickly lettuce.

In order to consider the potential prickly lettuce has as an alternative source for latex and rubber bioproducts, continued research and development must be conducted. The objectives of this research were 1) to compare rubber content and quality found in regional eastern Washington prickly lettuce biotypes and 2) to compare rubber content and quality of greenhouse verses field grown prickly lettuce plants.

**Materials and Methods**
Plant Material and Latex Component Isolation

Studies were conducted in 2009 at Washington State University’s Cook Agronomy Farm near Pullman, WA (GPS coordinates 46° 47' 3.555" N, -117° 5' 26.379" W) and concurrently in a greenhouse located on campus to evaluate the latex and rubber composition of 20 prickly lettuce biotypes. Seed of twenty eastern Washington prickly lettuce biotypes were collected in the fall of 2006 (Table 1). Plants were highly variable in growth habit and leaf morphology as determined by phenotypic evaluation published in previous work on the same biotypes (Riar et al. 2011). A single seed from each field collection was propagated in greenhouse conditions to obtain working stock seed collections from each biotype. Plants for the field and greenhouse studies were both started in greenhouse conditions by seeding working stock collections in 10 cm square plastic pots with potting media (LC1 Mix. Sun Gro Horticulture Distribution Inc., Bellevue, WA) and kept at 26 °C daytime and 22 °C night time (± 3 °C) temperatures. Natural light was supplemented with sodium vapor lighting to give a 14 h photoperiod that coincided with the daytime portion of the temperature profile. Plants were sub-irrigated as needed. Plants for the field study were transplanted to the Cook Agronomy Farm field site on May 3, 2009. Greenhouse grown plants were transplanted to larger 16 cm round plastic pots. No other inputs were used in the cultivation of either greenhouse or field plants.

The field trial area was prepared by fall chisel plowing followed by late-April cultivation and harrowing prior to transplanting. Latex samples were collected from non-treated plots in an unrelated field experiment studying herbicide tolerance which was arranged in a randomized complete block design. Plants were spaced 1 m from each other and from plants in the adjacent plot. Soil type was classified as Palouse silt loam (fine, silty, mixed, mesic, Pachic Ultic Haploxerolls). Soil pH was 5.2, and organic matter was 3.5%. Latex was collected twice from
greenhouse grown plants and four times from field grown plants. Because of the laborious nature of harvesting latex samples, collection was made over time rather than in space and each sampling time was treated as a replicate. Latex collections began once stems had developed through seed set. To collect latex, multiple diagonal cuts to plant stems were made with a razor blade and the resulting latex was collected into a tared micro-centrifuge tube. Tubes were capped and chilled, then re-weighed to obtain weight of initial latex collection. The latex collections were dried for 48 h under vacuum at 35 °C to remove all water. Tubes were re-weighed to acquire total water content. Dried samples were extracted three times with acetone by addition of one mL acetone, vortexing, centrifugation for five min at 6822 g, and collection of supernatant into a clean, tared vial. Acetone was dried under a stream of air and vials re-weighed to yield the total contained acetone soluble fraction (resin). Following acetone extraction, samples were extracted with hexane to dissolve rubber. One mL hexane was added to each tube followed by vortexing and hand stirring with a spatula to dislodge rubber pellet. Samples were centrifuged and supernatant collected into a clean tared vial. Rubber was extracted three times with hexane, the third hexane addition being allowed to sit 8-10 h before supernatant collection. Hexane was dried under air and vials weighed for total contained rubber. Original collection tubes containing insoluble latex material were dried and weighed to find the percentage of insoluble material.

**Rubber Analysis**

The rubber fraction was analyzed by gel permeation/size exclusion chromatography (GPC/SEC) HPLC, with refractive index detector to evaluate rubber (polyisoprene) polymer chain length. Dried rubber fractions were re-eluted in tetrahydrofuran (THF) to a concentration of approximately 2 mg mL⁻¹. Rubber was allowed to dissolve at room temperature overnight. A 200 µL portion of the dissolved rubber solution was removed from the top, without disturbing
any non-dissolved material at the bottom of the vial, and transferred to an HPLC vial with a 250 µL glass insert. 100 µL of the solution was injected by an autosampler. Samples were analyzed with an Agilent 1100 series HPLC equipped with an Agilent G1362A refractive index detector. Polyisoprene polymers were separated with a Phenogel Linear/Mixed Guard Column (10 µM 7.5x50 mm), and a PLgel Mixed-B size exclusion column (10 µM 7.5x300 mm) using THF as mobile phase. Run time was 17 min with a flow rate of 1 mL min⁻¹. Column and detector were maintained at 40 °C. Molecular weights were extrapolated using polystyrene standards from Varian (EasiVial) to create a semi-logarithmic standard curve. A single standard of 1,4-polyisoprene (Mw 999,000 g mol⁻¹) (Polymer Standards Service) was added to the standard curve as a check against the polystyrene polymers. Agilent GPC software was used together with standards to generate a standard curve and estimate average molecular weight (Mn), weighted average molecular weight (Mw), and D.

Statistical Analysis

Data were expressed as percent of total extract by weight and were subjected to ANOVA using PROC MIXED procedure in SAS (Version 9.2. SAS Institute, Inc., Cary, NC; SAS 2008). Type III statistics were used to test for significance (P ≤ 0.05) of site, biotype, and their interactions for latex composition including water, insoluble, acetone soluble, and hexane soluble components. Data were pooled across sites when no significant effects for site were detected. Comparison of pooled means for Mw, D, and percent rubber were subjected to ANOVA using PROC GLM procedure in SAS.

Results and Discussion

Latex was observed in all prickly lettuce biotypes collected for this study. Exuding latex was found in cambial regions located near the outer portions of stems and leaf veins from bolting
plants. *Lactuca* spp. laticifers are described as articulated, with an anastomosing vascular network developing initially in the procambium and later associating with the vascular cambium (Kekwick 2001; Hagel, Yeung, and Facchini 2008). Latex component analysis, calculated on a w/w basis, of the twenty biotypes revealed that on average, prickly lettuce latex is composed of 61.7% water, 23.3% insolubles, 13.8% acetone soluble (resin), and 3.2% hexane soluble material (rubber) (Figure 1). The amount of extractable rubber material between biotypes ranged from 2.2% to 4.9% contained by weight and had significant variation (*P* 0.0198) (Table 1). By comparison, Brazilian rubber tree latex is composed of 30 to 50% rubber; guayule 3 to 12% rubber and Russian dandelion latex varies from trace amounts to 30% rubber (Van Beilen and Poirier 2007). The latter two species continue to see improvements in extractable rubber due to intensive breeding strategies. Lettuce breeding has generated numerous cultivars selected mainly for palatability with no consideration of rubber producing traits. Prickly lettuce rubber content is also subject to improvement through rubber attribute screening and selection.

Analysis of variance (ANOVA) indicated no significant differences in the rubber collected under greenhouse conditions and field conditions of 2010. Rubber was collected at bolting stages in both environments, suggesting that rubber production may be more closely linked to growth stage than to specific environmental influences. Field condition in 2010 may have closely resembled those of the greenhouse environment, confounding observation of any potential environmental differences. Guayule, a perennial shrub, had greater accumulation of rubber when harvested after winter then after summer (Downes and Tonnet 1985). Analysis of prickly lettuce rubber in the bolting stage, grown under various environmental regimes would elucidate the influence environment has on rubber production and quality. Field assessments in a
variety of locations would also indicate the importance environment has on prickly lettuce rubber.

Molecular weight of the cis-1,4-polyisoprene isolated from rubber producing plants is an indicator of its end-use potential. To produce high performance rubber products with the desired elasticity, tensile strength, and abrasion resistance, the molecular weight must meet or exceed $3 \times 10^5 \text{ g mol}^{-1}$ (Swanson, Buchanan, and Otey 1979). Commercial grade rubber tree rubber exceeds $1 \times 10^6 \text{ g mol}^{-1}$. Molecular weight analysis of the twenty biotypes was performed on two latex collections of greenhouse grown plants and four collections of field grown plants (Table 1). Results were similar to that found by Bushman (2006) who compared prickly lettuce accession (UG92G488) to cultivated lettuce cv. Salinas (UC96US23) and found that both species produce high molecular weight rubber in bolting plants. In this study, all biotypes had an average polymer molecular weight suggesting high quality natural rubber ($>1 \times 10^6 \text{ g mol}^{-1}$). Additionally, several biotypes were found that consistently produce polymers in excess of $2 \times 10^6 \text{ g mol}^{-1}$ (Table 1). These biotypes would hypothetically have the highest quality rubber for manufacturing purposes. Statistical comparison of $M_W$ between biotypes revealed that there was significant variability between the biotypes collected ($P \ 0.0018$). Lettuce in general appears to have the biochemical mechanisms in place to consistently produce high molecular weight rubber as verified in this study and that of Bushman et al. (2006). Bushman described transgressive molecular weight values from a recombinant inbreed line (RIL) population of cultivated lettuce cv. Salinas x prickly lettuce (Argyris et al. 2005; Bushman et al. 2006). Transgressive molecular weight values observed in the RIL population were suggested to be due to genetic, developmental, or environmental influences. Biotypes with high molecular weight rubber found in this study may be genetic variants, containing a favorable allele set. Alternatively, it may be
that the high Mw Washington biotypes are trending towards optimal environmental conditions for high Mw rubber biosynthesis. However, because there were no differences in the rubber collected in greenhouse versus field grown plants, genetic controls as opposed to environmental cues appear to have a more significant influence on rubber production in prickly lettuce. Further studies must be conducted to determine if resultant molecular weight is due to genetic factors, environmental factors or a combination of such.

Polydispersity index (PDI), officially termed dispersity (D) (Stepto 2009), is another determinant of rubber quality. The value estimates overall dispersion of macromolecular species in the sample matrix. Dispersity is determined as a ratio of the weighted average molecular weight divided by the number average molecular weight (M_w/M_n). The closer the ratio is to one, the more homogeneous the polymer mixture. Average D for all biotypes and collections in this experiment was 2.91 (Table 1). Between biotypes, D ranged from 2.31 to 3.99, however ANOVA suggested D was not significantly different (P 0.062) (Table 1). Previous D values for cultivated lettuce and prickly lettuce were reported to be 1.1, indicating that the rubber polymers analyzed were nearly all the same length (Bushman et al. 2006). Differences between the studies may be due to the nature of collection and rubber processing methods. Bushman extracted and analyzed rubber from washed rubber particles, while in this study whole latex was directly extracted. Collection and washing of rubber particles may have stabilized the particles or enriched for a certain particle size and Mw whereas extracting from whole dried latex may have captured all potential polymers present or introduced degradation. In either case, D values for the rubber collected would be considered acceptable for the production of high-quality products. Brazilian rubber tree D values range from 2-11 and increase over the lifetime of the tree (Kovuttikulrangsie and Sakdapipanich 2004)
Conclusions

Prickly lettuce is a well-adapted weed that has potential as a source of natural rubber. Molecular weights of isolated cis-1,4-polyisoprene indicates this species has the ability to synthesize rubber ideal for high quality products. Overall content of rubber in latex is low compared to other species studied however improvements are expected with concerted breeding efforts, as seen in other species. Continued research will determine the utility of prickly lettuce as a rubber source. Development of plant harvest methods, rubber extraction methods, yield per hectare values, and rubber end-use quality assessment, will all need to be addressed in order to validate prickly lettuce as an economic rubber crop.

Although significantly studied, the mechanism of polyisoprene polymerization and chain length determination is not understood. Prickly lettuce and closely related cultivated lettuce are excellent model organisms for the study of rubber biosynthesis. Lettuce is easily transformed (Michelmore et al. 1987; Mazier et al. 2004; Lelivelt et al. 2005; Kanamoto et al. 2006) is amenable to gene-tagging (Mazier et al. 2007) and is a diploid, facultative self-pollinator that can be easily out-crossed (Nagata 1992). Recently, the lettuce genome has been released and is, to this author’s knowledge, the first released genome of a laticifer bearing, rubber producing plant. If deemed uneconomical as a rubber source, study of the rubber synthesis mechanisms in prickly lettuce could lead to a better understanding of rubber production in plants. Additionally, genes necessary for prickly lettuce rubber biosynthesis could be incorporated into more tradition crops species or low molecular weight rubber bearing plants.

Acknowledgements

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Literature Cited


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Table 1. Location of collected prickly lettuce biotypes and their averaged rubber qualities and quantity.

<sup>a</sup> Dispersity value defined as M<sub>W</sub>/M<sub>n</sub>, Where M<sub>W</sub> equals the polymer weight average molecular weight and M<sub>n</sub> equals the polymer number average molecular weight.
Figure 1. Average percentages of components found in tapped prickly lettuce latex from twenty eastern Washington prickly lettuce biotypes. Latex collected from both greenhouse and field grown plants and averaged.
Chapter 3

Quantitative Trait Loci Associated with Rubber Production in Prickly Lettuce

Abstract

Alternative sources of natural rubber are of great interest due to economic, biological, and political threats that could diminish supply of this essential resource. Prickly lettuce (*Lactuca serriola* L.) synthesized long chain natural rubber. Genotypic analysis was conducted on an F2 segregating prickly lettuce population to discover genetic regions linked to natural rubber production. A total of 461 EST-SSR derived primers were screened against the crossed parents. Of the screened markers, 89 polymorphic markers were selected and used to create a genetic linkage map. Three markers were not linked to any linkage group and five dominant markers did not map leaving 81 mapped markers in 12 linkage groups. Seventeen phenotypic measurements were taken from four main categories, latex extract components, rubber qualities, leaf measurements, and growth patterns. Interval mapping (IM) and multiple QTL mapping (MQM) identified several QTL in the mapping population that had significance based on LOD score thresholds. The trait, rubber $M_W$, had a QTL on Grp 8 near markers WSULs-21, WSULs-374.2, and WSULs-210. Dispersity had significant linkage to an unmapped marker, WSULs-374.1. Leaf perimeter and leaf lobing, similar traits, both had QTL on Grp 8 near markers WSULs-102 and WSULs-212. Stem counts and growth habit both had a similar QTL on Grp 4 near marker WSULs-304. Herbivory which was scored as plants that had been grazed or not grazed, most likely by deer, had a surprising QTL on Grp 8 between markers WSULs-12 and WSULs-21. The discovered QTL and the corresponding local markers are genetic resources for understanding rubber biosynthesis in prickly lettuce and could be used in marker assisted selection (MAS) breeding. Prickly lettuce is an excellent candidate for elucidating rubber synthesis mechanism and has potential as a crop plant for rubber production.
Introduction

Natural rubber (cis-1,4-polyisoprene) is a plant derived bio-product that has strategic importance due to the number of products that utilize the polymer. Commercial quantities of natural rubber are supplied by the Brazilian rubber tree [Hevea brasiliensis (Willd. ex A. Juss.) Müll. Arg.] a native of the Amazon basin. Yearly rubber harvest has steadily increased since rubber was first commercialized and in 2011, worldwide natural rubber production reached 10,978,000 t with consumption closely following at 10,919,000 t. Alternative sources of natural rubber are being investigated for their utilization in rubber production (Mooibroek and Cornish 2000; Van Beilen and Poirier 2007) to alleviate the threat of supply shortages. Prickly lettuce (Lactuca serriola L.), is one of the few rubber bearing plant species able to produce high M_W rubber (Bushman et al. 2006) yet to date, no research has been conducted to identify genetic influences of rubber biosynthesis or molecular markers linked to rubber trait development.

Prickly lettuce is closely related to, and most likely the progenitor of, cultivated lettuce (Lactuca sativa L.). It is believed the two species are conspecific (Koopman, Zevenbergen, and Berg 2001) and are easily crossed to produce viable offspring. Cultivated lettuce is grown throughout the world and consumed for its nutritious foliage (Mou 2012; Nicolle et al. 2004). Cultivated lettuce has 9 chromosomes and a genome size of approximately 2600 Mb. Prickly lettuce also has 9 chromosomes and presumably a similar genome size. The Compositae Genome Project Database (CGPDB; http://compgenomics.ucdavis.edu) database is the most comprehensive genetic resource for study of the compositae family including cultivated and four closely related lettuce species, L. serriola L., L. saliva L., L. virosa L. and L. perennis L., ordered in proposed phylogenetic relatedness to cultivated lettuce. The genetic and genomic architecture of cultivated lettuce and its relatives has been well documented and includes a large
inventory of molecular markers. Variable marker types include RFLPs (Kesseli, Ochoa, and Michelmore 1991; Kesseli, Paran, and Michelmore 1994), RAPDs (Waycott and Fort 1994), ISSRs (Vicente et al. 2008), genomic/EST-SSRs (Riar et al. 2011; Simko 2009; Van de Wiel et al. 2010), AFLPs (Jeuker et al. 2001; Jansen et al. 2005; Koopman, Zevenbergen, and Berg 2001), APs (Hu et al. 2005), SNPs (Moreno-Vázquez et al. 2003) and SFPs (Van Leeuwen et al. 2009). The developed molecular markers have been effectively utilized for linkage mapping (Truco et al. 2007), gene-tagging, QTL mapping (Jeuker et al. 2001) and genetic diversity studies (Kuang et al. 2008; Riar et al. 2011). Although cultivated lettuce is the main research focus, many of the molecular markers are transferable and have been used between lettuce species. Most recently the lettuce genome has been released (www.lgr.genomecenter.ucdavis.edu) and will become a valuable asset in lettuce research as well as close lettuce relatives through syntenic genetic comparisons.

Prickly lettuce is important in lettuce breeding as donors of disease resistance genes (Lebeda 1998; Lebeda, Pink, and Astley 2002; Reinink 1999; Ryder 1999; Sicard et al. 1999; Zohary 1991) insect resistance (Ellis et al. 2002; Kishaba et al. 1980; Mou and Liu 2004), trait development such as carotenoid content (Mou 2005), bolting time (Ryder and Milligan 2005) and water use efficiency (Johnson et al. 2000). On the basis of the gene-pool concept (Harlan and Wet 1971), Lactuca species were categorized into three gene pools based on their relationship to cultivated lettuce (McGuire et al. 1993). The primary gene pool is represented by numerous cultivars of cultivated lettuce, primitive landraces, and wild species where crossing barriers do not exist. Prickly lettuce is the primary representative as the progenitor species of this gene pool (de Vries 1997) and will be the greatest beneficiary of cultivated lettuce genetic advancements.
Prickly lettuce is a potential new crop plant for rubber production. Development of this species will require molecular tools that assist in breeding efforts for desired traits. Of equal scientific importance are the general mechanisms controlling rubber biosynthesis in all rubber bearing plants. There are few studies that attempt to find genetic associations to rubber traits and no studies in prickly lettuce. The purpose of these studies were (1) to understand the genetic control of rubber traits in prickly lettuce and (2) identify molecular markers or QTL that would lead to a better understanding of the genes involved in rubber biosynthesis and allow for selection of prickly lettuce biotypes that have increased rubber making potential.

**Materials and Methods**

**Plant Material and Population Development**

In 2006, twenty eastern Washington prickly lettuce biotypes were collected and later screened for rubber content and quality. Previous screening of the collected biotypes for herbicide resistance and latex rubber revealed two with distinct rubber content and quality traits as well as contrasting visual phenotypes (Figure 1). Parent 6-9/13 (P1) had lobed leaves, was resistant to acetolactate synthase (ALS) targeting herbicides, was early to bolt, had average rubber M\(_w\) of 2.5x10\(^6\) g mol\(^{-1}\), and contained 4.9 % rubber in the latex on average. Parent 4-10/6 (P2) had non-lobed oblanceolate leaves, no herbicide resistance, was late to bolt, an average rubber M\(_w\) of 1.1x10\(^6\) g mol\(^{-1}\), and contained an average of 2.2 % rubber in the latex. Parental plants were grown in a greenhouse from seed in 16 cm round plastic pots into potting media (LC1 Mix. Sun Gro Horticulture Distribution Inc., Bellevue, WA) with temperatures kept at 26 °C daytime and 22 °C nighttime (± 3 °C). Natural light was supplemented with sodium vapor lighting to give a 14 h photoperiod that coincided with the daytime portion of the temperature profile. Plants were sub-irrigated as needed. At anthesis, flowers were crossed using the clip-and-
wash method (Nagata 1992). Reciprocal crosses were made and F1 seed collected. F1 seed were planted and evaluated for potential self-pollination using the differential parental phenotypic traits of bolting time and leaf lobing, semi-dominant and dominant traits respectively. Successfully crossed F1 plants had an intermediate bolting time between parental phenotypes and inherited leaf lobing traits (lobed in 4-10/6 x 6/9-13 crosses) that allowed for selection of non-selfed individuals. Cross efficiency was 81% with 13 of 16 successful crosses. One F1 plant with cross 6-9/13 female x 4-10/6 male was selected for creation of an F2 segregating population by collecting seed from the selfed F1 plant.

**Phenotypic Evaluation**

In total, 250 F2 individuals were grown in a greenhouse from seed for four weeks prior to planting at Washington State University’s Cook Agronomy Farm Pullman near Pullman WA (GPS coordinates 46° 47' 1.0854", -117° 5' 28.755"). Plants were started May 7, 2010 in a greenhouse under the same conditions as the parental and F1 plants but were planted in smaller 10 cm square plastic pots. Plants were transplanted to the field June 7, at 2 m row spacing with 1 m distance between each plant. Two plants died leaving a total of 248 individuals in the population. Throughout the growing season phenotypic data was collected from four main categories including, latex extract components, rubber qualities, leaf measurements, and growth patterns. Flowering time phenotype was confounded by deer herbivory, however deer appeared to graze preferentially on certain individuals within the population. Plants were scored with either herbivory or no herbivory. Lobing was scored as either lobed or not lobed. Leaf area and leaf perimeter values were calculated by scanning four fully expanded leaves on a flatbed scanner and using the ImageJ (Rasband 1997) convert to mask and wand tool functions to outline each leaf. Stem number was determined by counting the number of stems on each plant at 90 d
after planting. Growth habit was scored on a scale of one to five where one represented the 6-9/13 parental phenotype which consists of early bolting and multiple lateral shoots and five represented 4-10/6 parental phenotype that had a late bolt timing and few lateral shoots.

Latex from each individual was collected from bolting stem for latex component and biochemical analysis. The percent of water, resin, rubber and insoluble material from each plant was determined gravimetrically (% wt/wt). Latex was collected by making multiple diagonal incisions with a razor and catching the drips of latex into a tared microcentrifuge tube. Tubes were capped and re-weighed to obtain weight of initial latex collection. Latex was then dried for 48 h under vacuum at 35 °C to remove all water. Tubes were re-weighed to acquire total contained water. Dried samples were extracted three times with acetone by addition of one mL acetone, vortexing, centrifugation for five min at 6822 g, and collection of supernatant into a clean, tared vial. Acetone was dried under a stream of air and vials re-weighed to determine resin content. Following acetone extraction, samples were extracted with hexane to dissolve rubber. One mL hexane was added to each tube followed by vortexing and hand stirring with a spatula to dislodge the rubber pellet as needed. Samples were centrifuged and supernatant collected into a clean tared vial. Latex was extracted three times with hexane, the third hexane addition being allowed to sit 8-10 h before supernatant collection. Hexane was dried under air and vials weighed for total contained rubber. Original collection tubes containing insoluble latex material were dried and weighed to find the percentage of insoluble material.

The rubber fraction was further analyzed by high performance liquid chromatography gel permeation/size exclusion chromatography (HPLC GPC/SEC), with refractive index (RI) detection to evaluate rubber (polyisoprene) polymer chain length. Dried rubber fractions were re-eluted in tetrahydrofuran (THF) to a concentration of approximately 5 mg mL⁻¹. Rubber was
allowed to dissolve at room temperature overnight. A 100 µL portion of the dissolved rubber solution was removed from the top, without disturbing any non-dissolved material at the bottom of the vial, and transferred to an HPLC vial with a 250 µL glass insert. An autosampler injected 40 µL of the dissolved rubber solution. Samples were analyzed with an Agilent 1100 series HPLC equipped with an Agilent G1362A refractive index detector. Polyisoprene polymers were separated with a Phenogel Linear/Mixed Guard Column (10 µM 7.5x50 mm), and a PLgel Mixed-B size exclusion column (10 µM 7.5x300 mm) using THF as mobile phase. Run time was 17 min with a flow rate of 1 mL min\(^{-1}\). Column and detector were maintained at 40 °C.

Molecular weights were extrapolated using polystyrene standards from Varian (EasiVial) to create a semi-logarithmic standard curve. A single standard of 1,4-polyisoprene (\(M_W\) 999,000 g mol\(^{-1}\)) (Polymer Standards Service) was added to the standard curve as a check against the polystyrene polymers. Agilent GPC software was used together with standards to generate a standard curve and estimate average molecular weight (\(M_n\)), weighted average molecular weight (\(M_W\)), and \(\bar{D}\). The values for polyisoprene characterization were determined by selecting the earliest eluting peak (longest polymer). Low \(\bar{D}\), a beneficial trait, is independent of polymer \(M_W\) but rather reflects the \(\bar{D}\) of the selected peak. In this study, the earliest eluting peak may have low \(M_W\) and low \(\bar{D}\).

Phenotypic values for the entire population were plotted verses frequency to generate histograms (Figures 2 and 3). Analysis of phenotypic frequencies over the population determined if traits were single gene controlled having expected ratios of 3:1, 1:2:1 or multi-genic (quantitative) with a normal distribution. Suspect single gene traits were examined by Chi-square analysis (\(X^2\)) and quantitative traits analyzed for QTL (Tables 1 and 3).

**Prickly Lettuce Genetic Map**
DNA from each individual in the F2 population and parents was extracted from young leaves that were frozen (-80 °C) and freeze dried. Extraction was performed on approximately 30 mg dried tissue using a Biosprint 96 DNA Extractor following manufacturer protocols. DNA concentrations were measured with a Nanodrop 2000 spectrophotometer and normalized to 50 ng µL⁻¹. A total of 400 EST-SSR derived primers that were previously mined from the CGPDB (CGPDB; http://compgenomics.ucdavis.edu) to analyze prickly lettuce genetic diversity were used for polymorphic screening (Riar et al. 2011). An additional 61 EST-SSR primers developed in 2009 (Simko 2009) were added giving a total of 461 EST-SSR’s available to screen against the crossed parents. Polymorphism of the mined EST-SSR’s was carried out using two genotyping platforms. The first employed 10 % polyacrylamide gel electrophoresis (PAGE) denaturing gels with silver stain to visualize amplified fragments. Polymerase chain reactions (PCR) were carried out in 20 µL total volume mixtures each containing 100 ng template DNA, 0.5 µm SSR primers, 250 µM dNTPs, 1.5 mM MgCl₂, 0.5x PCR buffer and 1.0 U Taq DNA polymerase (MangoTaq, Bioline USA Inc.). Reactions were carried out on a Techne TC-4000 thermocycler with the following PCR profile: initial denaturation at 95°C for 3 min followed by 40 cycles at 95 °C for 30 sec, 57 °C for 30 sec, 72 °C for 25 sec, and a final extension at 72 °C for 10 min.

Of the 461 primers 380 were further analyzed by a second genotyping platform utilizing an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). PCR amplifications were carried out in 12 µL reaction mixture each containing 15 ng template DNA, and three PCR primers [M13F primer (5′-CACGACGTGTTAAAAACGAC-3′) labeled with one of 4 fluorescent dyes (6-FAM, PET, VIC, or NED; Applied Biosystems, Foster City, CA), long forward primer with M13F-tail at its 5′ end and a reverse primer]. Primer concentrations were 0.25 µM reverse
primer, 0.20 µM M13F and 0.05 µM of the long, M13 tagged, forward primer. Other components included 200 µM dNTPs, 1.0 mM MgCl2, 1.0 x PCR buffer and 1.0 U Taq DNA polymerase (made in-house). PCR amplifications were done on Biorad C1000 or S1000 thermocycler using the following condition: initial denaturation at 95 °C for 3 min followed by 42 cycles at 95 °C for 40 sec, 57 °C for 60 sec, 72 °C for 60 sec, and a final extension at 72 °C for 10 min.

**Segregation Analysis/ Linkage Maps**

Initially, 89 polymorphic EST-SSR markers were selected and used to create a genetic linkage map (Table 2). Of the 248 individuals in the F2 population 186 were analyzed to facilitate 96 and 384 well formats. Fragment analysis of markers in the F2 population was carried out using an Applied Biosystems 3730XL DNA analyzer and GENE MARKER software version 1.5 (SoftGenetics LLC 2006). PCR conditions were the same as described for the parental screen in the second genotyping platform. Fragments were scored as either parent 1 (a) parent 2 (b) or heterozygous (h). Scored data was analyzed by JoinMap 4.0 (Van Ooijen 2006) using maximum likelihood algorithm with all other parameter set to default. The Haldane mapping function was used to calculate genetic distances. Maps were verified using Mapmaker 3.0b (Lander et al. 1987) at LOD score 3.0. Of the original 89 markers, three were not linked to any linkage group and five dominant markers did not map leaving 81 mapped markers in 12 linkage groups (Figure 4). The traits of leaf lobing and herbivory were scored bimodally as presence or absence phenotypes and analyzed by Chi square (Table 1).

**Polymorphism**
Marker polymorphism was determined as the difference in nucleotide base-pairs between parents as established by the ABI 3730XL DNA analyzer. Fragment sizes were estimated by comparison to a size standard added to each well and tagged with fluorescent dye LIZ.

**Trait Genetic Markers/QTL Analysis**

F2 genotypic and phenotypic data were analyzed using MapQTL 6.0 (Van Ooijen 2009) to assist in identifying marker-trait correlations. Analysis was done using both interval mapping (IM) to allow for possible correlations with the unmapped markers and multiple-QTL model (MQM). Default parameter for IM were used and included: algorithm-regression, test statistic-LOD, fit dominance for F2-yes, mapping step size-1.0, neighboring markers-5, number of iterations-200, functional tolerance-1.0x10^-08, permutation-1000. MQM model had the same default parameters. Chromosomes with associated QTL were made using MapChart 2.2 (Voorrips 2002) (Figure 4).

To determine the LOD score significance thresholds a permutation test of 10,000 iterations (p=0.05) was performed over each chromosome (Churchill and Doerge 1994). QTL were considered significant if the output over the chromosome for the trait of interest exceeded the minimum threshold.

**Results and Discussion**

**Phenotypic Assessment**

Phenotypes in the F2 population were quantitative in that they did not follow Mendelian segregation ratios with the exceptions of leaf lobing and low M_w. These traits segregated at a ratio of 3:1 ($X^2=0$ df 1) and ($X^2=2.6$ df 1) respectively (Table 1). Leaf lobing observed in this experiment is consistent with previous reports for inheritance of the trait where lobing was reported as dominant to non-lobed leaves and controlled by a single gene (Durst 1930, Whitaker...
Leaf area in the F2 population had a normal distribution with the median closer to that of parent 6-9/13 and very few individuals sharing a large leaf area phenotype similar to 4-10/6 (Figure 3). Leaf perimeter also had a less defined normal distribution pattern (Figure 2). The plant growth phenotypes stem number and growth habit had normal distributions with the majority of phenotypes intermediate between parents (Figure 2). Rubber D values over the population were normally distributed (Figure 3) and appear eschewed to higher D, although values cannot fall below the optimal D of one. Normal distributions were observed for all latex components including the amount of rubber, resin, insoluble material, and water contained in the latex (Figure 3). The M_W of the rubber from the F2 population was normally distributed however there was an enrichment of individuals with low M_W rubber (Figure 2). The reason for the elevated number of extremely low M_W phenotypes is unclear. Rubber from these individuals may have degraded at some point during collection, storage, or extraction. Alternatively, the individuals with low M_W rubber may have underlying genetic influences affecting polymer stability or determinant polymer length. Just under 25% of the F2 population had inferior, low M_W rubber (<100000 g mol⁻¹) suggesting the low M_W phenotype may be a single gene trait (Table 1). Repeated studies from a recombinant inbreed line (RIL) derived from the cross made for this study will assist in determining whether the low M_W phenotype is an artifact of sampling or a defined genetic trait.

**Linkage Maps**

The assembled linkage map generated from 81 EST-SSR markers produced 12 linkage groups or chromosomes. The chromosome number for lettuce and prickly lettuce is 9 (Babcock et al 1937). Discrepancies in chromosome number are most likely due to the low number of markers used in this study. A few of the markers have been mapped in the CGPDB and were
used as anchors for assigning chromosome names for the associated linkage group. Based on maps generated in the CGPDB, some of the linkage groups made by JoinMap 4.0 in this study had distances longer or shorter than would be expected (Figure 4). For example, Grp 1 is anchored by SML-41 and corresponds to chromosome 7 in CGPDB, MAP 2 JMR2. Chromosome 7 is 126.8 cM in length compared to 332.6 cM in this study. Grp 5 is anchored by markers SML-29 and SML-34 both on chromosome 9 of MAP 2 JMR2. Chromosome 9 is 102.9 cM compared to our 52.8 cM distance. Grp 6 is anchored by SML-26 to chromosome 2 which is 162.1 cM in MAP 2 JMR2, a much closer distance to 158.5 cM of this study; however SML-26 is placed near the middle of MAP 2 JMR2 at 72.0 cM while in our map SML-26 is nearer to the end at 148.7 cM. MapMaker 3.0b was used to verify grouping made by JoinMap. Mapmaker was more stringent in grouping of markers and further divided some of the linkage groups generated in JoinMap. The distinction is noted by bold, red markers designated MM3.0b placed half the distance between the flanking mapped markers in the chromosome groupings. In general, JoinMap 4.0 and MapMaker 3.0b grouped markers similarly, lending confidence to the map structure.

**Rubber Trait QTL**

Of particular interest in this study are the markers and QTL linked to rubber traits. One QTL and one unmapped marker had significant correlations to rubber traits, rubber $M_W$ and polymer D. Rubber $M_W$ had a QTL associated with Grp 8 near markers WSULs-374.2 and WSULs-21. The rubber $M_W$ QTL peaks at marker WSULs-374.2, at LOD 3.24, and accounted for 7.7% of the phenotypic variation in the population (Figure 4, Table 3). Dispersity is a measure of polymer homogeneity as measured by weighted average molecular weight divided by the number average molecular weight ($M_W/M_n$) (Stepto 2009). An optimal ratio of 1.0 signifies
uniform polymers. Dispersity is a beneficial trait if the rubber also has high M\_W. The marker WSULs-374.1 had significant linkage to D with a peak LOD value of 4.79 and explained 11.2% of the phenotypic variation (Table 3) as determined by IM. It is important to note that the D and M\_W values were based on the earliest eluting peak by HPLC-SEC/GPC and are therefore not necessarily correlated to one another. Low D does not mean high M\_W. Review of the D and M\_W values reveals that the majority of individuals with low D were more closely related to the low M\_W individuals in the population. Interpretation of the marker is counter intuitive and suggests that the higher D values are representative of a beneficial trait. Although high D is less desirable, nearly all of the D values collected from the earliest eluting peak would be considered acceptable for production purposes (Kovuttikurlangsie and Sakdapipanich 2004). The WSULs-374 markers are derived from the same primer yet generated distinct PCR products that segregated sufficiently to separate the two at a LOD threshold of 3.0. At LOD 2.0 WSULs-374.1 and WSULs-374.2 are grouped together; however at this threshold they are separated by 122.1 cM. Both the rubber M\_W QTL and D marker are derived from 6-9/13 alleles. This single marker could be used for selection and integration of prickly lettuce with acceptable D and high M\_W rubber, the two most important traits in determining rubber end-use potential (Swanson, Buchanan and Otey 1979).

**Other Beneficial Traits**

Cultivated lettuce cultivars have been developed to meet consumer and producer needs. Most have a delayed bolt time and, if allowed to bolt, have only a single stem. They produce copious leaf tissue and little latex during vegetative growth (Mou 2008). Many of these traits are in stark contrast to those desired in a rubber producing cultivar. Anecdotal evidence suggests latex production increases with bolt initiation (Bushman 2006). In this regard, early bolting with
multiple stems would both be beneficial traits for rubber lettuce production. A strong growth habit and stem count QTL was identified in our study on Grp 4 that is highly correlated to marker WSULs-304 (Figure 4). The QTL significance continues at a lower level over a group of adjacent markers WSULs-310, WSULs-84, WSULs-204, WSULs-383, WSULs-2 with the highest significance at marker WSULs-383 in this group. The growth habit phenotype was characterized by early bolt time with multiple lateral shoots. The growth habit QTL peaked at LOD 11.94 near marker WSULs-304 and LOD 2.98 near WSULs-383 and respectively explained 25.6 % and 7.1 % of the population phenotypic variation (Figure 4, Table 3). As expected, stem count is strongly related to growth habit as a numerical reflection of the growth habit phenotype. The stem count QTL also peaked at markers near WSULs-304 and WSULs-383 with respective LOD values 13.84 and 3.43 and phenotypic variances 29.0 % and 8.1 % (Figure 4, Table 3). Growth habit associated markers will assist in selection of rubber lettuce cultivars that bolt early and have many stems. These traits may also facilitate multiple harvests over the season, increasing rubber yields. Alternative to harvesting fast bolting multi-stem plants, crisphead lettuce cultivars that have high latex and rubber content would be ideal for current lettuce cultivation and harvest practices. Many of the head lettuce cultivars have high biomass (700 to 1000 g head\(^{-1}\)), disease resistance, and reduced chlorophyll content (Mou 2008). The crisphead lettuce cv. ‘Salinas’, released in 1975, is the most widely grown lettuce cultivar in the history of lettuce production (Mou 2008). Introggression of alleles that confer high rubber content and \(M_W\) in non-bolting head lettuce such as ‘Salinas’ lettuce leaf tissue would be perfectly suited for current cultivation practices. The identified markers and QTL are useful tools for MAS and introgression of those traits into new rubber lettuce varieties. Further selection of prickly lettuce
weedy traits such as water use efficiency, would allow rubber lettuce to be grown on marginal lands decreasing competition with existing lettuce production areas and other food crops.

In this study additional interesting marker-phenotype associations, not necessarily related to rubber lettuce development, have been identified. Leaf perimeter, a numerical expression of leaf lobing, and herbivory both had significant QTL on Grp 8 (Figure 3). As previously noted lettuce leaf lobing has been identified as a dominant, single gene trait (Durst 1930, Whitaker 1950). Leaf perimeter QTL is associated with markers WSULs-102 and WSULs-212 which had respective peak LOD values of 20.87 and 27.32 and explained 40.3 % and 49.2 % of the phenotypic variation for the population (Figure 4 Table 3). The histogram for leaf perimeter is not normal and may be more indicative of epistatic interaction. The QTL for the lobing phenotype was stronger than the perimeter trait with peak LOD values of 29.7 and 45.4 at respective markers WSULs-102 and WSULs-212. Population phenotypic variance for the lobing trait was 52.0 % near marker WSULs-102 and 67.5 % at WSULs-212. Leaf lobing is an important horticultural trait in cultivated lettuce (Michelmore 2009). On an evolutionary level, lobed leaves may impart greater hydraulic efficiency and heat dissipative properties (Nicotra et al. 2012; Sack and Tyree 2005). There are no reports of a leaf lobing gene in lettuce however a single large effect QTL has been identified on chromosome 3 through analysis of the core F7:8 RIL mapping population derived from a cross between L. sativa cv. ‘Salinas’ and L. serriola accession UC96US23 (Michelmore 2009). The QTL found in this study is most likely the same found by Michelmore (2009) placing Grp 8 markers on chromosome 3 of the lettuce map. Based on sequence similarity, the EST related to marker WSULs-102 has resemblance to the Arabidopsis thaliana cyclin family protein AT5G67260 (CYCD3; 2) (Table 2). In general, cyclin proteins act as transcription factors activating cyclin-dependent kinases during the cell cycle in
turn regulating key transitions during mitosis (Jackson 2008). In Arabidopsis, CYCD3; 2 is expressed in the shoot apical meristem and persists in young leaves. CYCD3; 2 functions by determining cell number in developing lateral organs and mediating the effects of cytokinin on apical growth and development (Dewitte et al. 2007). Leaf morphogenesis in tomato was dependent on cytokinin levels and overexpression of a cytokinin biosynthesis gene IPK7 caused increased expression of CYCD3 (Shani et al. 2010). The EST for WSULs-212 has similarity to AT4G14960 tubulin alpha-6 chain (TUA6) (Table 2). TUA6 encodes an isoform of tubulin necessary for right handed helical growth (Abe and Hashimoto 2005). It is interesting to find that both EST-SSRs markers associated with the leaf perimeter and lobing QTLs are involved in plant organ morphogenesis. Further study of these genes in lettuce would determine if either of the genes is the causal agent for leaf lobing in lettuce. Overexpression of the genes in a non-lobed lettuce background could help identify these candidate genes as the leaf lobing gene in lettuce.

A prickly lettuce herbivory QTL was found on Grp 8 and peaked at LOD 3.25 near marker WSULs-374.2. The population phenotypic variance for the QTL was 7.7% (Figure 4 Table 3). The phenotype was observed as preferential feeding of bolted stems in the F2 population by white tail deer (*Odocoileus virginianus* Zimmermann.). There is considerable evidence suggesting latex is a beneficial adaptation to deter predation (Agrawal and Konno 2009; Konno 2011). One example in lettuce was found where latex from an insect resistant cultivar ‘Valmaine’ prevented *Diabrotica balteata* (LeConte.) beetles from feeding of latex painted lima bean leaves, however feeding was not inhibited when latex from the susceptible cultivar ‘Tall Guzmaine’ was applied (Huang el al. 2003). A follow-up study showed that differential compounds in the semi-polar latex extract contained the inhibitory element(s) (Sethi
et al. 2008). Prickly lettuce contains an abundance of the sesquiterpine lactones lactucin and lactucopicrin (Sessa et al. 2000). Increase in the levels of these compounds was correlated to perceived bitterness by sensory analysis (Price et al. 1990). The compounds also have sedative and analgesic affects (Wesołowska et al. 2006). Chemical analysis of the resin extracts containing the sesquiterpines compared with plant feeding patterns will help determine if these compounds act as a deer herbivory deterrents or stimulants.

Conclusions

In summary, a set of polymorphic EST-SSRs have been used to generate a linkage map from a cross between two distinct eastern Washington prickly lettuce biotypes. Analysis of the map with phenotypic data has led to the discovery of QTL and markers useful in development of rubber lettuce cultivars. The identified QTL and corresponding local markers could be used in a MAS breeding scheme to find individuals with high $M_w$ rubber, and multi-bolting traits or introgression of rubber producing traits into current lettuce cultivars. Importantly, the QTL will allow further investigation of the genes that control these traits now that linked chromosomal regions have been identified. Additional QTL and markers related to lettuce leaf morphology and herbivory could be used in lettuce breeding for horticultural traits and understanding leaf ontogeny or lend evidence to latex herbivory relationships.

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Deven See, Jamin Smitchger and Randall Stevens from Department of Crop and Soil Sciences, Washington State University for their technical assistance in lab and field studies.
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Figure 1. Leaf morphologies of the parents selected for generation of F2 segregating population.
Table 1. Chi square analysis of potential prickly lettuce single gene traits derived from F2 population of cross between eastern Washington biotypes 6-9/13 x 4-10/6. Leaf lobing followed a perfect 3:1 ratio ($X^2=0$ df 1) herbivory deviated from 3:1 ($X^2=4.8$ df 1), while low $M_W$ was statistically within 3:1 ratio ($X^2=2.6$ df 1).
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<th>Reverse primer (5'-3')</th>
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**Notes:**
- AT5G05210-nucleolar matrix protein-related, contains Pfam domain, PPO435: Surfeit locus protein 6
- AT1G22450-cytochrome c oxidase subunit 9b, putative (COXb)
- AT4G18370-protease HboA, chloroplast (SPPA) (HHOA)
- AT2G46680-homocobras-leucine zipper protein 7 (HB-7)
- Unknown
- AT5G17020-exportin1 (XPO1)
- AT3G63030-methyl-CpG-binding domain-containing protein
- AT2G14910-expressed protein
- AT5G09900-UDP-glucosyltransferase family protein
- AT5G58580-similar to nitrate-responsive NOX protein
- AT5G53300-ubiquitin-conjugating enzyme 10 (UBC10)
- AT5G60170-RNA recognition motif (RRM)-containing protein
- AT2G41120-expressed protein
- TaG01010-rac GTPase activating protein
- AT2G20980-auxin-responsive AUX/IAA family protein
- AT5G48655-zinc finger (C3HC4-type RING finger) family protein
- AT5G60760-cyclin family protein
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*M13 tail sequence added to forward primer, ‘5-CACGACGTTGTAAACGAC-3’

Table 2. Polymorphic EST-SSR markers used for map development and QTL analysis of an F2 population derived from a cross of the eastern Washington prickly lettuce biotypes 6-9/13 x 4-10/6.
Figure 2. Distribution of selected traits showing QTL on Grp 4 and Grp 8 from an F2 population derived from a cross of eastern Washington prickly lettuce biotypes 6-9/13 x 4-10/6.
Figure 3. Distribution of phenotypic traits from an F2 population derived from a cross of eastern Washington prickly lettuce biotypes 6-9/13 x 4-10/6.
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<td>3.24</td>
<td>2.2</td>
<td>223844</td>
<td>145724</td>
<td>6-9/13</td>
</tr>
<tr>
<td>Dispersitya</td>
<td>WSULs-374.1</td>
<td>11.2</td>
<td>4.79</td>
<td>NA</td>
<td>0.94</td>
<td>-0.27</td>
<td>6-9/13</td>
</tr>
<tr>
<td>Leaf perimeter</td>
<td>8, WSULs-102</td>
<td>40.3</td>
<td>20.87</td>
<td>2.2</td>
<td>19.15</td>
<td>4.97</td>
<td>6-9/13</td>
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<tr>
<td></td>
<td>8, WSULs-212</td>
<td>49.2</td>
<td>27.32</td>
<td>2.2</td>
<td>21.09</td>
<td>9.12</td>
<td>6-9/13</td>
</tr>
<tr>
<td>Lobing</td>
<td>8, WSULs-102</td>
<td>52.0</td>
<td>29.68</td>
<td>2.2</td>
<td>0.37</td>
<td>0.32</td>
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<tr>
<td></td>
<td>8, WSULs-212</td>
<td>67.5</td>
<td>45.39</td>
<td>2.2</td>
<td>0.42</td>
<td>0.41</td>
<td>6-9/13</td>
</tr>
<tr>
<td>Herbivory</td>
<td>8, WSULs-374.2</td>
<td>7.7</td>
<td>3.25</td>
<td>2.2</td>
<td>-0.07</td>
<td>-0.17</td>
<td>6-9/13</td>
</tr>
<tr>
<td>Growth habit</td>
<td>4, WSULs-304</td>
<td>25.6</td>
<td>11.94</td>
<td>2.5</td>
<td>-0.92</td>
<td>0.04</td>
<td>6-9/13</td>
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<tr>
<td></td>
<td>4, WSULs-383</td>
<td>7.1</td>
<td>2.98</td>
<td>2.5</td>
<td>-0.36</td>
<td>0.41</td>
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<td>Stem count</td>
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<td>13.84</td>
<td>2.6</td>
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<td>1.07</td>
<td>6-9/13</td>
</tr>
<tr>
<td></td>
<td>4, WSULs-383</td>
<td>8.1</td>
<td>3.43</td>
<td>2.6</td>
<td>1.62</td>
<td>0.92</td>
<td>6-9/13</td>
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*a Unmapped marker, non-QTL, single marker association as determine by IM.

Table 3. QTL and associated markers
Figure 4. Linkage groups and QTL from eastern Washington prickly lettuce biotype cross (6-9/13 x 4-10/6). Group designations derived from JoinMap 4.0 software. Bold, red makers with designation MM3.0b are further separation of linkage groups determined by MapMaker 3.0b. Chromosome designations assigned based on mapped EST markers found in CGPDB and are underlined. The bold markers SML-60 and WSULs-372 were derived from the same EST contig but found in independent SSR mining experiments. Significant QTLs are shown next to linkage groups as black bars with a 1 and 2 LOD QTL interval and corresponding QTL LOD graph.
Chapter 4

Post-Harvest Content, Quality and Physical Properties of Rubber Derived from
Prickly Lettuce

Abstract

The latex bearing plant prickly lettuce (*Lactuca serriola* L.) was studied to determine extractability of rubber and evaluate rubber molecular and physical characteristics after extraction. Post-harvest chemical extraction methods of rubber from freeze dried, milled, whole plants and latex extracted rubber from living plants was evaluated. All post-harvest chemical extraction methods on whole plant material yielded small quantities of rubber that was of low quality while direct rubber extraction from latex yielded high quality rubber. Nuclear magnetic resonance spectroscopy (NMR) indicated the presence *cis*-1,4-polyisoprene, with little to no *trans*-polyisoprene present. Latex-extracted rubber had physical properties similar to those of Brazilian rubber tree (*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.) making it an excellent source of rubber for manufacturing.

Introduction

Natural rubber (*cis*-1,4-polyisoprene) is a vitally important plant-derived bioproduct essential in the manufacture of over 40,000 products including medical devices (Mooibroek and Cornish 2000). Rubber tree, a tropically endemic species is the source of industrialized natural rubber production. Harvest of natural rubber has steadily increases since it was first discovered and commercially produced. Worldwide manufacture of natural rubber in 2011 reached 10,978,000 million t with consumption at 10,919,000 t in 2011. Yearly production is at demand levels with minimal surpluses reported. In 2010, there was a 377,000 t deficit in consumption versus production (International Rubber Study Group 2012). Natural rubber has strategic
importance given that the current source of natural rubber is endemic to tropical regions. Security of supply chains for countries were the rubber tree cannot be cultivated is a high priority. Plant species that are able to efficiently generate the quantity and quality of natural rubber as their tropical counterparts has yet to be found.

The ideal rubber-producing crop for temperate regions will be a fast growing annual that produces large amounts of biomass. Annual crops are more easily incorporated into crop rotation systems and can be planted or removed in response to market fluctuations and independent grower requirements (Van Beilen and Poirier 2007). Perennial plant species such as trees or shrubs often have higher starting costs, have a lag from input to harvest and remain in place for several growing seasons, making them less suited to changing market demands. Preferably, the rubber producing species will also be tolerant and well adapted to cultivation on marginal land, alleviating competition with food crops, as seen in corn-ethanol production (Babcock and Fabiosa 2011). The required tolerance and adaptability traits are often present in weedy species. Weeds are well adapted to the region they infest, require low production inputs, have high water-use efficiency, and are largely resistant to disease and insect pests. Many of these species have high photosynthetic efficiency and consequently high biomass yields or are drought resistant. In some cases, populations of herbicide resistant weeds may provide production options for their development as a crop. Weedy plants could provide value-added bioproducts that increase the economic feasibility of establishing them as crops for biomass and bioproducts. Prickly lettuce (*Lactuca serriola* L.) is one such weed, and has been identified as a plant species able to produce high molecular weight rubber (Bushman et al. 2006).

Interestingly, the genus *Lactuca*, of which prickly lettuce is a prominent member, has long been recognized as a potential source of rubber (Bushman et al. 2006). In 1913, a report
detailed the analysis of latex from two lettuce species, *Lactuca canadensis* L. and *L. scariola* L. (Fox 1913). Both species secreted latex from all areas of the plant. Rubber content of the latex was measured at 2.2 % in *L. canadensis* and 1.6 % in *L. scariola*. The variability of rubber content and the ability to produce rubber among lettuce genotypes was also described by the Clemson Agriculture Center (Mitchell, Rice, and Roderick 1942) and the US Department of Agriculture (Polhamus 1958). The reports described differences in latex rubber content of several lettuce species with negligible differences in percent rubber extracted from whole plants. None of these reports discussed the purity or composition of the latex, the molecular weights of the rubber in the latex, or the condition of the tissues previous to extraction – that work was completed by Bushman et al. in 2006.

Bushman et al. (2006) examined prickly lettuce and a cultivated lettuce cultivar (*Lactuca sativa* L. cv. Salinas) to determine characteristics of natural rubber in those two lettuce species. They demonstrated that both species produce latex containing desirable high molecular weight (>1 million g mol$^{-1}$) *cis*-1,4-polyisoprene with very narrow polydispersity that should lead to excellent product characteristics after vulcanization. Bushman et al. (2006) also concluded that genetic, developmental, or environmental influences could impact latex molecular weight and suggested optimizing the genetic, physiological, and environmental conditions for the synthesis of higher molecular weight rubber for prickly lettuce. In order to elucidate the potential prickly lettuce has as an alternative source for latex and rubber bioproducts, further research and development must be conducted.

Evaluation of rubber from twenty eastern Washington prickly lettuce biotypes collected in 2006 found that all biotypes had rubber considered high quality as determined by molecular weight and dispersity (D). Variation was observed in rubber content and quality with some
biotypes producing exceptionally high molecular weight rubber. The collection serves as a source for understanding several prickly lettuce biological characteristics including weed resistance (Burke et al. 2009), genetic diversity (Riar et al. 2011) and in this work, rubber production potential. In preliminary screening of rubber content and quality, rubber was extracted directly from tapped latex. As an annual plant with relatively small stems, extracting rubber from tapped latex would not be feasible in large scale production. Rubber extraction from whole plants would be a more realistic approach to harvesting prickly lettuce rubber.

Rubber is an elastomer that possesses unique chemical and physical properties. Polyisoprene in its original state is elastic, waterproof, and moldable. Yet native polyisoprene has limited use capabilities. At low ambient temperatures it becomes brittle, while at high temperatures tackiness and increased malleability becomes problematic. The basis for these properties is independent movement of the isoprene polymers in relation to one another. Polymer chain entanglements are the only interacting junctures to hold shape (Mark and Erman 1994). It was not until the discovery of vulcanization that the true potential of rubber was realized. Vulcanization introduces cross-linkages of the isoprene polymers mainly through sulfur bridges. Sulfur cross-linking is achieved when rubber dienes react with sulfur in the presence a catalyst, under heat and pressure. Vulcanization increases the retractile force of distorted polymer chains when under mechanical stress and reduces the amount of permanent deformity remaining when deforming force is removed (Mark and Erman 1994).

Although vulcanized rubber is the final end product, physical properties of pre-vulcanized rubber (green rubber) are important indicators of rubber end-use performance. Thermal properties or physical state changes are one gauge of the polymer qualities. Detection of energy changes and heat capacity as the material transforms from solid to amorphous to liquid
states provides the thermal properties of glass transition temperatures (Tg) and melting temperature (Tm). The Tg of a material is one indicator of the end use potential, specifying the temperature at which hardening occurs. Materials cannot be processed or worked or becomes brittle once below the Tg (Sichina 2000). The Tg also relates to the end use properties. For example, thermosetting two-part epoxy resin has a Tg below 25 °C until mixed. Once combined, cross linking of the resin while curing raises the Tg well above room temperature creating a hard bonding material (Sichina 2000). Tensile strength, another physical property, is defined as the resistance of an applied load in the opposite direction. As a pulling force is applied, tension forces cause the polymers to align, reducing the number of chain entanglements (Blyler 1969; Hamed 1981). More chain entanglements impart a stronger resistive tensile strength. The tensile strength of a polymer increases with higher molecular weight (more entanglements) and low D (fewer short chains). In addition to chain entanglements, green strength of non-vulcanized elastomers is influenced by branching (Blyler 1969; Grechanovskii, Ivanova, and Poddubnyi 1973), polar group interactions (Grechanovskii, Ivanova, and Poddubnyi 1973), gel content (Grechanovskii, Ivanova, and Poddubnyi 1973; Grechanovskii, Poddubnyi, and Ivanova 1974), crystallization on stretching (Grechanovskii, Poddubnyi, and Ivanova 1974), and test sample preparation (Amnuaypornsri, Sakdapipanich, and Tanaka 2009). Green strength of non-vulcanized rubber is important as it determines end-use processability, especially in mass production systems. Molded non-vulcanized tires would lose their shape while waiting to be vulcanized without proper green strength (Hamed 1981).

To utilize plant synthesized rubber, intact particles or freed polymers must be tapped or extracted from the plant. In larger woody tree species such as Brazilian rubber tree, tapping is the method of choice. Tapping of rubber tree latex is usually done once every 2-3 days for 9 months.
of the year. Collection is made by cutting a thin layer of bark just above the cambial layer and collecting drips of latex into a cup. Anti-coagulating agents, usually ammonia, are added during collection to preserve the latex in liquid form or formic acid is added to the latex to coagulate the suspended rubber particles and collected as cup lump rubber (Sakdapipanich and Rojruthai 2012). Other rubber producing species under commercial investigation are not amenable to tapping procedures: therefore more destructive methods must be used to release and then isolate rubber particles or polymers from plant tissue. Plant processing in this manner presents challenges in maintaining rubber integrity throughout the extraction process. For example, processing of bulk rubber from guayule (Parthenium argentatum Gray.) has been difficult due to low bulk viscosity and entrained co-extracted resin causing oxidative and thermal polymer degradation (Schloman 2005). The chemical and physical properties of rubber isolated from plant tissue is an artifact of the polymer structure within the latex-bearing cells and the method of polymer extraction as noted in guayule extractions (Schloman 2005).

In general there are two main methods of rubber extraction, solvent extraction and flotation. Chemical extraction of rubber entails isolation of rubber polymers from plant material and other metabolites using selective solvents after grinding of plant material. Solvents can be added sequentially or simultaneously. In sequential solvent extraction, a polar organic solvent such as acetone is first used to extract pigments, polar lipids, resins, and other polar metabolites while leaving non-polar hydrocarbons. A second, non-polar dissolving solvent such as hexane, cyclohexane, toluene or benzene is used to then solubilize rubber. All other non-polar compounds such as waxes and lipids could be present as impurities in the rubber fraction. Simultaneous solvent extraction includes addition of a single solvent such as xylene (Wagner and Parma 1988) or an azeotropic mixed solvent system (Beinor and Cole 1986). Simultaneous
extraction produces a mixture of dissolved resin and rubber. Precipitation of the rubber by addition of acetone or alcohol is a means of further purifying the rubber polymers. For most small scale analytical purposes, chemical extraction has been the method of choice. Researchers have used a mixture of chemical extraction methods to analyze rubber from various plant derived sources (De Rodriguez and Kuruvadi 1991; Hammond and Polhamus 1965; Mekkriengkrai et al. 2004; Nurthen et al. 1986; Pearson et al. 2010; Pearson, Cornish, and Rath 2013; Spanò et al. 2012). However, on a large scale, chemical extraction is not the method of choice due to costs associated with solvent purchase and recovery and flammability hazards. Rubber from guayule has been extracted for bulk rubber production through chemical extraction methods (Hamerstrand and Montgomery 1984; Schloman 2005).

Flotation extraction methods have also been used in lab scale experiments and bulk rubber production. This method usually involves milling and centrifugation steps in an aqueous buffer, the addition of acid to coagulate rubber or creaming agents to float the rubber particles is a common practice (Buranov and Elmuradov 2010; Cornish and Backhaus 1990). Direct water, milling and floatation methods have been developed for application on fleshy plants (Eskew and Edwards 1946; Stamberger, Koenig, and Hanslick 1946)

The efficient extraction of rubber from a highly complex plant matrix continues to be a problem in all facets of natural rubber production. Brazilian rubber tree tapping of latex bypasses impurities introduced from whole plant extraction. However, tree tapping and latex collection is performed by hand, decreasing efficiency, and latex stability must be maintained by addition of anti-coagulants and anti-oxidants. Plants that must be destroyed to harvest rubber need refinements to extraction procedures that increase rubber yield and decrease rubber deterioration.
Efficient, non-degradative extraction of rubber from prickly lettuce will be a major hurdle to overcome to produce high quality bulk rubber.

To make high-quality, high performance vulcanized rubber products the natural rubber starting material must meet certain criteria. Polymer chain length or molecular weight and molecular weight distribution (dispersity) have a direct relationship to the processability of the rubber (Swanson, Buchanan, and Otey 1979). In general, longer polymer chains with narrow D, give the best end-use performance.

The end-use potential of plant isolated rubber is dependent on the quality of rubber obtained post-extraction. Methods of harvesting, storage and extraction affect the quantity and quality of rubber available to make rubber products. To date, there are no studies evaluating extraction methods or physical properties of prickly lettuce derived rubber. The objectives of these studies were (1) to evaluate latex extracted rubber compared to extraction methods on dried prickly lettuce whole plant material (2) assess the rubber quantity, quality and molecular characteristics post extraction and (3) determine if prickly lettuce rubber has physical properties suitable for the rubber industry.

**Materials and Methods**

**Plant Material Origin**

In the fall of 2006 seed from twenty eastern Washington prickly lettuce biotypes was collected. A single seed from each field collection was propagated in greenhouse conditions to obtain working stock seed collections from each biotype.

**Plant Material for Latex Extraction**

Stock seed from six biotypes, including the two used in whole plant extractions (Table 1), were used to extract rubber from latex. Plants were grown at Washington State University in a
greenhouse located on campus and simultaneously at Cook Agronomy Farm near Pullman, WA (GPS coordinates 46° 47' 3.555" N, -117° 5' 26.379" W). Plants for both locations were started under greenhouse conditions by seeding working stock seed in 10 cm square plastic pots with potting media (LC1 Mix. Sun Gro Horticulture Distribution Inc., Bellevue, WA). The greenhouse was kept at 26 °C daytime and 22 °C night time (± 3 °C) temperatures. Natural light was supplemented with sodium vapor lighting to give a 14 h photoperiod that coincided with the daytime portion of the temperature profile. Potted plants were placed in trays and sub-irrigated as needed. After four weeks, plants for field study were transplanted to the Pullman site. The field trial area was prepared by fall chisel plowing followed by late-April cultivation and harrowing prior to transplanting. Latex samples were collected from non-treated plots in an unrelated field experiment studying herbicide tolerance which was arranged in a randomized complete block design. Plants were spaced 1 m from each other and from plants in the adjacent plot. Soil type was classified as Palouse silt loam (fine, silty, mixed, mesic, Pachic Ultic Haploxerolls). Soil pH was 5.2, and organic matter was 3.5 %. Latex collections began once stems had developed through seed set. Greenhouse plants were transplanted to taller 16 cm round plastic pots. No other inputs were used in the cultivation of either greenhouse or field plants.

**Plant Material for Whole Plant Extraction**

Two biotypes were evaluated for whole plant rubber extraction, biotypes 6-9/13 and 4-10/6 (Table 1). The selected biotypes were chosen based on preliminary screening of the original twenty collected biotypes which revealed they had the most distinct rubber producing properties. Stock seed was sown in 10 cm square pots with potting media (LC1 Mix Sun Gro Horticulture Distribution Inc., Bellevue, WA) under greenhouse conditions. Plants were kept at 26 °C daytime and 22 °C night time (± 3 °C) temperatures. Natural light was supplemented with
sodium vapor lighting to give a 14 h photoperiod that coincided with the daytime portion of the temperature profile. Plants were sub-irrigated as needed. After approximately four weeks in the greenhouse, plants were transplanted to a field location near Pullman Washington at Washington State University’s Cook Agronomy Farm near Pullman. The 2010 site (GPS coordinates 46° 47’ 3.555” N, -117° 5; 26.379” W) was planted May 22 while the 2011 site (GPS coordinates 46° 46’ 55.6284” N, -117° 9.96” W) had a much later planting date of June 7 due to cold and wet weather conditions. Plantings were at 2 m row spacing with 1 m between each plant. Four individual plants representing four replicates were used in the study.

Both biotypes were harvested at or near anthesis. For biotype 6-9/13 this was 75 and 79 d after planting in 2010 and 2011 respectively. Biotype 4-10/6 was harvested at 140 d in 2010 and in 2011 at 119 d after planting. The 2011 harvest of biotype 4-10/6 was prior to reaching anthesis due to threat of frost. Plants were harvested by cutting the stem at ground level and placing in a paper bag. Wet weights were immediately taken and plants were frozen and stored at -80 °C. Whole frozen plants were crushed by hand and lyophilized using an FTS Systems Multi-Dry (Model FD-5-84A-D). Dry weights were taken to determine percentage of water loss. Freeze dried plant material was crushed and homogenized with a Wiley mill and stored in a sealed plastic bag at 4 °C until extraction.

**Latex Extraction**

Latex was collected twice from greenhouse grown plants and four times in the field. Latex collections began once stems had developed through seed set. To collect latex, multiple 45° angular cuts to plant stems were made with a razor blade and the resulting latex was collected into a tared micro-centrifuge tube. Tubes were capped and chilled. The latex collections were dried for 48 h under vacuum at 35 °C to remove all water. Dried samples were
extracted three times with acetone by addition of one mL acetone, vortexing, centrifugation for five min at 6822 g, and collection of supernatant into a clean, tared vial with teflon lined cap. Acetone extraction was followed by hexane extraction to dissolve rubber. One mL hexane was added to each tube followed by vortexing and hand stirring with a spatula to dislodge rubber pellet. Samples were centrifuged and supernatant collected into a tared vial. Rubber was extracted three times with hexane, the third hexane addition being allowed to sit 8-10 h before supernatant collection. Hexane was dried under air and vials were re-weighed for total contained rubber.

**Whole Plant Rubber Extraction and Yield**

Three rubber extraction methods for dried, powdered prickly lettuce were compared. The first utilized a 1 L capacity soxhlet apparatus and a two solvent system. A known amount (~50 g) of crushed and homogenized plant material was placed into a single thickness cellulose thimble, 60 mm x 180 mm (Whatman). A folded piece of cellulose filter paper was placed on top of the sample to prevent splashing of dripped solvent. Acetone was used as an initial solvent to remove pigments and resin. Acetone (500 mL) was poured into the lower collection flask and 5 to 10 teflon boiling stones were added. The soxhlet apparatus was assembled and the lower flask heated to an even boil. Condenser liquid was maintained at 10 °C. Acetone extraction was continuously run for 48 h. after which time extraction solvent was transferred and dried under a stream of air into a tared vial. Extracts were further oven-dried at 55 °C for 24 h. Dried acetone extract was re-weighed to yield total percent resin. Samples were subjected to a second, non-polar solvent to extract rubber. In a separate clean flask 500 mL hexane and boiling stones were added. Soxhlets were heated and allowed to reflux for 48 h. Hexane extract was transferred to a
tared vial similar to the acetone extract however no oven drying occurred. The dried hexane extract was re-weighed to yield total contained percent rubber by weight.

The second extraction method employed the same two solvent system, however plant material went through a solvent slurry and filtration procedure. A known amount (~50 g) of dried, milled plant material was placed into a 500 mL Erlenmeyer flask. The first solvent, acetone, was added to completely cover the powder (200 mL). The antioxidant butylated hydroxytoluene (BHT) was added at 0.1 % wt/wt (1.0 mM). The acetone slurry was further homogenized and pulverized with a Polytron homogenizer for 60 s. A stir bar was added to the flask and allowed to mix for 24 h. The resultant slurry was vacuum filtered with a Buchner filter funnel through three coffee filters into a 1 L filter flask. Filter cake was washed with acetone. Filterate was successively transferred to a tared vial and dried under a stream of air. Further drying was done in an oven at 55 °C for 24 h. Acetone extract was re-weighed to determine total percent resin. The filter cake was left in the filter and oven dried for 16 h. at 55 °C then removed and cooled to room temperature. Rubber was extracted in the second solvent phase. Dried, acetone extracted powder was weighed into a 500 mL Erlenmeyer flask. Hexane was added to cover the powder. The sample flask was capped and stirred slowly for 48 h. The slurry was vacuum filtered in the same manner as the acetone slurry and washed with copious amounts of hexane. Hexane filtrate was transferred to a tared vial and dried under a stream of air. As a final rinse, each flask was washed with 30 mL tetrahydrofuran. Dried hexane extract was re-weighed to yield total contained hexane soluble material.

In the third method of extraction, plant material was extracted via direct hexane solvent slurry followed by alcohol precipitation to recover rubber. A known amount of plant material (~50 g) was added to a 500 mL Erlenmeyer flask and 200 mL hexane was added to cover the
powder. Antioxidant (BHT) was added at 0.1 % wt/wt. Each sample was capped and allowed to sit without stirring at room temperature for 5 d to dissolve rubber. The hexane slurry was vacuum filtered and washed with hexane. An equal volume of ethanol and methanol were added to the filtrate to precipitate the rubber fraction. The solution was allowed to sit for 6 d to precipitate all rubber. Precipitate was collected by vacuum filtering the solution through a 0.2 µm nylon filter. Filters with precipitate were placed into a vial with 20 mL hexane to re-dissolve rubber material. Each filter was washed three times with 20 mL hexane. Washes were allowed to sit 24 hours, centrifuged at 2800 g and clear solvent removed into a tared vial. Hexane was evaporated under a stream of air and vials re-weighed to determine contained hexane soluble precipitate.

**Rubber Molecular Weight, Physical Properties and NMR**

To analyze rubber polymers characteristics, hexane soluble fractions from whole plant extractions and latex extractions were analyzed by high performance liquid chromatography gel permeation/size exclusion chromatography (HPLC-GPC/SEC). Polymers were detected with refractive index (RI) detection to evaluate rubber (polyisoprene) polymer chain length. Dried hexane soluble fractions were re-eluted in tetrahydrofuran (THF) to a concentration of approximately 5 mg mL\(^{-1}\). Samples were allowed to dissolve at room temperature overnight. A 100 µL portion of the dissolved rubber solution was removed from the top, without disturbing any non-dissolved material at the bottom of the vial, and transferred to an HPLC vial fitted with a 250 µL glass insert. An autosampler injected 70 µL of the solution. Samples were analyzed with an Agilent 1100 series HPLC equipped with an Agilent G1362A RI detector. Polyisoprene polymers were separated with a Phenogel Linear/Mixed Guard Column (10 µM 7.5x50 mm), and a PLgel Mixed-B size exclusion column (10 µM 7.5x300 mm) using THF (BHT stabilized) as
mobile phase. Flow was set to 1 mL min$^{-1}$ with a run time of 17 min. The column and detector were heated to 40 °C. Sample molecular weights were extrapolated using Varian (EasiVial) polystyrene standards to create a semi-logarithmic standard curve. A single standard of 1,4-polyisoprene ($M_W$ 999,000 g mol$^{-1}$) (Polymer Standards Service) was added to the standard curve as a check against the polystyrene polymers. Agilent GPC software was used together with standards to generate a standard curve and estimate average molecular weight ($M_n$), weighted average molecular weight ($M_W$), and $D$. Rubber material isolated from direct latex extraction was analyzed for physical properties. All other extraction procedures on dried homogenized plant material yielded low molecular weight rubber by HPLC/GPC and were therefore unsuitable for analysis (Table 2).

Samples for NMR and physical properties analysis were combined by biotype and further purified. THF stored samples from the six collection times were pooled together. Combined samples were centrifuged at 2800 g for 15 min and the top clear solution was removed to a tared vial, repeated three times with 10 mL fresh THF. Samples were concentrated under air to approximately 10 mL. Acetone was used to precipitate and purify rubber polymers. Acetone was added (30 mL) to vial capacity and the solution was air dried again to 10 mL and 30 mL of fresh acetone was added to the vial. Rubber precipitate was centrifuged at 2800 g, solvent decanted and washed twice with fresh acetone.

For dynamic mechanical analysis (DMA) combined, precipitated rubber samples were dissolved in hexane and cast by floatation. A rectangular mold dimensions 3.2 x 2.1 x 0.5 mm was filled with 3 mL water and dissolved rubber sample was pipetted to the top of the water surface and allowed to air dry at room temperature. Films were carefully removed and held between glass slides with a few drops of water to prevent sticking. Films were cut to an average
width of 3.5 mm and had an average thickness of 113.5 µm. Cut films were clamped into a TA Instruments Q800 DMA to test viscoelastic properties such as tensile strength. The instrument was set to perform a controlled force experiment at 28 °C with a ramp force of 0.1 N min⁻¹. Stress-strain curves were generated to determine Young modulus (slope of linear range before yield), and maximal yield force.

Differential scanning calorimetry (DSC) was performed using a TA Instruments Q200 instrument with 5-10 mg of the combined precipitated rubber. Samples were subjected to two heating and cooling cycles beginning at -90 °C and heated to 200 °C at a rate of 10 °C min⁻¹. For analysis purposes, the first heating curve was omitted and the second curve studied for thermal properties.

Identity of the extracted hexane soluble fractions was determined by ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy using a Varian 400 Mhz. Combined precipitated rubber samples from prickly lettuce biotype 6-10/6 and the synthetically prepared polyisoprene standard were used for NMR structure determination. The samples were dissolved at 1% (w/v) in CDCL₃ and heated to 50 °C during analysis.

Results and Discussion

Latex Rubber Extraction and Yield

Rubber extracted directly from latex yielded more rubber of higher quality than any of the other tested methods on dried powdered prickly lettuce material. On average the amount of contained rubber in latex ranged from 2.2 to 4.9 % wt/wt (Table 1). Molecular weight the rubber extracted from latex was indicative of high polymer quality. Average M₇₀ ranged from 1119222 to 2175798 g mol⁻¹ with Ð values between 2.26 and 2.68 (Table 1).

Whole Plant Rubber Extraction and Yield
Extraction from homogenized whole plant material was undertaken in an attempt to simulate scale-up potential for prickly lettuce rubber production. Post harvest freezing followed by freeze drying and grinding was thought to be least destructive to rubber polymers while allowing for solvent extraction. However, the extraction methods used on this material yielded very little rubber all of which was low molecular weight (Table 2). Soxhlet extraction yielded approximately 0.04 % w/w rubber from biotype 6-9/13 in both the 2010 and 2011 studies with average $M_W$ of 29931 and 38491 g mol$^{-1}$ respectively. Biotype 4-10/6 yielded slightly more rubber, 0.093 % (2010) 0.064 % (2011), however the quality was inferior with $M_W$ at 918 and 4140 g mol$^{-1}$ in 2010 and 2011. The hexane slurry followed by alcohol precipitation yielded 0.046 and 0.022 % w/w in 2010 and 2011 from biotype 6-9/13 with $M_W$ of 17236 and 187330 g mol$^{-1}$, respectively. Biotype 4-10/6 precipitation amounts were nearly indistinguishable and insufficient for GPC analysis. The two solvent slurry extract demonstrated higher relative yield, yet the hexane extract had wax-like properties, presumably due to contamination. Polymer characterization via chromatography was not undertaken.

Rubber from prepared samples had degraded during post-harvest processing or in the extraction steps. The double bond diene groups of rubber polymers are susceptible to oxidative attack by free radicals. It is likely the presence of oxidative compounds released in the solution during chemical extraction degraded the rubber polymers as this degradation was not observed in rubber extracted from directly-tapped latex. Partitioning of the plants further by stem or root may help alleviate the degradation effects. Unfortunately, latex from root tissue was not examined in this study although cut taproots reveal the presence of latex in root organs. Rubber extracted from root tissue using the chemical extraction methods presented may be of higher quality as there are fewer oxidative compounds present during the extraction process.
NMR Analysis

Analysis of proton and carbon NMR spectra confirmed the presence of cis-1,4-polyisoprene in the selected test samples. Distinctive proton chemical shifts indicating presence of the olefinic proton (H-C=C) at 5.13 ppm, the methylene proton (-CH$_2$-C=C) at 2.06 ppm and the cis-methyl proton (CH$_3$-C=C) 1.68 at ppm (Figure 1). Characteristic carbon shifts were observed consisting of the two ethylenic carbons (-CH$_2$-C=C-CH$_2$) at 135.1 and 125.0 ppm, the methylenic carbons (-CH$_2$-C=C-CH$_2$) at 26.3 and 32.1 ppm and the methyl carbon (CH$_3$-C=C) at 23.3 ppm (Figure 2). Little to no trans-polyisoprene was detected as carbon signals corresponding to trans-methyl (16.0 ppm) and trans-methylene (40.0 ppm).

Physical Properties

DMA measures viscoelastic properties as material is deformed under controlled stress and temperature. One important viscoelastic property is tensile strength or green strength when referring to non-vulcanized compounds. The green strength of the prickly lettuce samples tested had Young’s modulus ranging from 0.0037 to 0.0166 MPa and maximal yield points between 0.1678 and 0.2849 MPa (Table 1, Figure 3). By comparison, maximal yield point of rubber tree rubber is reported to be 0.25 MPa (ASTM 2009).

DSC studies phase transition of a non-crystalline or semi-crystalline material when heated or cooled. The prickly lettuce rubber samples tested had Tg’s ranging from -64.15 to -64.95 °C (Table 1, Figure 4). As a comparison the Tg of natural rubber isolated from Brazilian rubber tree is -75 °C (National Institute for Materials Science). Molecular weight and D, reflected in the number-average molecular weight (Mn), are factors contributing to the Tg of polymeric material (Claudy et al. 1983). As Mn increases, Tg also increases to a maximal Mn to Tg value. The relationship is expressed by the Fox and Flory equation, $T_g = T_g(\infty) - K_g/M_n$.
where Tg(∞) is the limiting Tg at very high Mn and Kg is the free volume constant (T. G. Fox and Flory 1950). The polyisoprene standard had the highest Tg of -62.08 °C corresponding to the highest Mn, 951428 g mol⁻¹. Five of the rubber samples tested had average Mn values near 800,000 g mol⁻¹ (± 87,000) all of which had a Tg ranging from -64.15 to -64.95 °C (Table 1). Biotype 4-10/6 had a lower Mn of 470261 g mol⁻¹ which corresponded to a Tg of -65.05 °C (Table 1).

Melting point is another physical transition state in polymers, where a material changes from a crystalline phase to a solid amorphous phase. The melting point determines the maximum workable temperature of a material. The prickly lettuce rubber samples had a melting point temperature (Tm) ranging from 47.66 °C to 59.07 °C. For comparison, the Tm of rubber tree rubber is estimated to be near 41 °C (National Institute for Materials Science).

The thermal properties of prickly lettuce rubber were similar to or better than that of rubber tree rubber, the industry standard. The high molecular weight and low Đ of the prickly lettuce rubber attributed to high Tg, Tm, and green strength.

Conclusions

Natural rubber isolated from the common weed prickly lettuce is a potential rubber source. Isolation of rubber directly from latex yielded polymer of high quality as revealed by high Mw and low Đ. The rubber exhibited exceptional physical properties similar to rubber tree rubber. The extraction of rubber from whole plant material as described in this study proved to be suboptimal giving very low yields and degraded rubber polymers. Optimization of post-harvest treatment and extraction methods will need to be determined in order for prickly lettuce to become a suitable domestic source of natural rubber.

Acknowledgements
The authors would like to acknowledge funding for this project through the *Aegilops cylindrica* – Biomass for Biofuels and Bioproducts from Weedy Plants (NIFA/USDA special grant). Special thanks go to Randall Stevens, Misha Manuchehri, and Dennis Pittman, from the Department of Crop and Soil Sciences, Washington State University for, their technical assistance in lab and field studies. Assistance with NMR analysis was given by Bill Hiscox at the Washington State University NMR Center. NMR equipment was supported by NIH grants RR0631401 and RR12948, NSF grants CHE-9115282 and DBI-9604689 and the Murdock Charitable Trust. Rubber physical property analysis was performed with the guidance and support of Dr. Armando McDonald and Liqing Wei at the University of Idaho, Renewable Materials Program College of Natural Resources.
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National Institute of Materials Science (NIMS), Polymer database Polymer ID (P060002)

http://polymer.nims.go.jp/PoLyInfo/cgi-bin/pi-id-search.cgi?PID=P060002&pred=on

(Accessed January 21, 2013)


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Polyisoprene stdᵃ NA NA NA NA NA 999000 951428 1.05 -62.08 NA NA NA

ᵃ Synthetic 1,4-polyisoprene standard purchased from Polymer Standards Service. M_w, M_n and D obtained from manufacturer and T_g determined experimentally.

ᵇ Weighted average molecular weight. Values were weighted again based on mass of each combined individual.

ᶜ Number average molecular weight.

d Dispersity value defined as M_w/M_n, Where M_w equals the polymer weight average molecular weight and M_n equals the polymer number average molecular weight.

e Glass transition temperature as analyzed by DSC.

ᶠ Melting point temperature as analyzed by DSC.
Table 1. Location of collected prickly lettuce biotypes and their averaged rubber qualities, quantity, and physical properties found in extracted latex.
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<sup>a</sup> Hexane extraction step in two solvent slurry extraction contained large amounts of wax material that cannot be attributed to rubber content.

Table 2. Amount of extractable material and rubber M<sub>W</sub> using different extraction methods from whole, freeze dried and ground prickly lettuce plants on a percent wt/wt basis.
Figure 1. Proton (^1H) NMR spectra for latex extracted acetone washed, combined and precipitated prickly lettuce rubber from biotype 6-10/6.
Figure 2. Carbon ($^{13}$C) NMR spectra for latex extracted acetone washed, combined and precipitated prickly lettuce rubber from biotype 6-10/6.
Figure 3. Dynamic mechanical analysis of green strength on acetone washed, precipitated natural rubber isolated from 4 biotypes of prickly lettuce.
Figure 4. Differential scanning calorimetry curves of synthetic isoprene rubber and prickly lettuce natural rubber.
Chapter 5

Microscopic Characterization of Rubber Particles in Prickly Lettuce

Abstract

Prickly lettuce contains high molecular weight polyisoprene and has potential as an alternative source of natural rubber. Rubber producing plants synthesize and store polyisoprene in sub-cellular rubber particles. In most rubber bearing plants rubber particles are contained in specialized vascular cells known as laticifers. To further characterize prickly lettuce rubber producing capabilities, rubber particles were isolated and measured by scanning electron microscopy. Isolated, fixed, gold coated rubber particles were viewed by Field Emission Scanning Electron Microscopy (FESEM). Most of the observed WRPs had an average diameter of 0.288 µm (SE ±0.005) and ranged in size from 0.88 µm to 0.443 µm. Fewer, larger particles were also observed ranging from 0.575 µm to 4.9 µm in diameter, however they were isolated individuals. Prickly lettuce particles have a similar size range as Euphorbia lactiflua Phil. or the intermediate size particles of Brazilian rubber tree [Hevea brasiliensis (Willd. ex A. Juss.) Müll. Arg.].

Introduction

Natural rubber is one of the most important plant derived bioproducts. The tropically endemic rubber tree is the single source of industrialized rubber production, a cause for concern should any problems arise in rubber production or export. To stem rubber supply concerns, alternative sources of natural rubber are being investigated (Mooibroek and Cornish 2000; K. Cornish 2001; Van Beilen and Poirier 2007; Van Beilen and Poirier 2007) with a short list of species able to synthesized high molecular weight rubber. Prickly lettuce (Lactuca serriola L.)
was identified as a high molecular weight rubber producer (Bushman et al. 2006) yet little is known on its rubber making ability.

Prickly lettuce has the same or similar anatomical features as other rubber producing plants. Rubber is made and stored in specialized vascular cells called laticifers. Laticifer producing plants have either articulated laticifers where cells are connected via anastomoses, as with prickly lettuce, or are non-articulated (Kekwick 2001). Guayule (Parthenium argentatum Gray.) does not possess laticifers but rather produces latex in specialized bark parenchyma cells (Ross 1908). Within the cytoplasm of laticifers cells or guayule parenchyma cells are found spherically shaped, organelle-like, rubber particles (Backhaus and Walsh 1983; Schmidt et al. 2010; Singh et al. 2003; Wood and Cornish 2000). Rubber particles are surrounded by a fatty-acid mono layer membrane imbedded with the enzymatic machinery necessary for isoprene transfer and polymer elongation within the hydrophobic core of the particle (Cornish 2001; Nawamawat et al. 2011). The protein and lipid components found in laticifer membranes are species specific. Within species, the lipid membranes appear to be composed of the same lipid components of the cells from which they are derived. Similar compositions suggest the absence of a highly conserved structural and chemical composition of particle membranes (Cornish 2001).

In this study, the size and structure of prickly lettuce rubber particles is described by electron microscopy. Because rubber particles are the central point of enzymatic synthesis of rubber, understanding their morphology in comparison to other known particle bearing species is essential to understanding rubber biosynthesis.

**Materials and Methods**

**Isolation of Washed Rubber Particles**
Freshly tapped latex was dripped into centrifuge tubes containing chilled buffer solution consisting of 100 mM Tris-HCl, 5 mM MgSO₄, and 5 mM DTT in distilled water. The buffer collection solution was pH adjusted to 7.5 using 0.5 N sodium hydroxide. Adjustment of pH is critical in that complete degradation of particles was observed when buffer was not slightly basic. Latex was centrifuged at 915 g for 10 min to pellet particles, and supernatant was discarded. Particles were washed three times with isolation buffer and then stored in buffer at 4 °C.

**Fixing and Viewing of Rubber Particles**

Prior to fixing, WRPs were viewed with a light microscope at 40x magnification to verify the presence and integrity of isolated particles (Figure 1). For comparison purposes, particles from Indian rubber tree (*Ficus elastica* Roxb. ex Hornem.) were isolated and viewed in tandem (Figure 1). WRPs were initially fixed with 3 % glutaraldehyde in 50 mM sodium cacodylate and 1% tannic acid at room temperature for 1 h. Fixed particles were washed three times in 50 mM sodium cacodylate then post fixed in 1% osmium tetroxide (OsO₄) for 18 h. Fixed particles were washed three times with distilled water and stored in distilled water. Post fixing, particles were viewed under light microscope again to verify particle integrity during the fixing process. Suspended particles were placed on a cover glass mounted on a stub and allowed to air dry. Samples were gold coated using a Hummer V sputter coater before viewing by field emission scanning electron microscopy (FESEM). Samples were viewed using an FEI Quanta 200F FESEM instrument under high vacuum.

**Results and Discussion**

Rubber particles isolated and fixed in this study were spherical in shape, a common shape in all studied rubber particles (Cornish et al. 1999; Singh et al. 2003; Wood and Cornish 2000).
The average diameter was 0.288 µm (SE ±0.0056, n=144) and particle size ranged from 0.088 µm to 0.443 µm (Figure 2, 4). Larger particles were also observed that ranged in size from 0.575 µm to 4.9 µm (Figure 3). These particles however, were isolated individuals and did not represent the major fraction of those observed by FESEM. It is worth noting that there appears to be a significant amount of particle collapse and degradation - coagulated and coalesced rubber pieces were observed forming a lattice like structure (Figure 2 B, C). This degradation likely occurred during particle isolation as some larger coagulated rubber was present when viewed under light microscopy (Figure 1 A), prior to fixing. Small intact particles accumulated at the edges of the liquid phase and around coagulated rubber pieces during the air drying of the fixed particles (Figure 1 B). It is possible that the larger rubber particles were less stable and represent the portion of coagulated rubber; however no particles of this size were observed under light microscopy. The observation of larger particles agrees with particle size distribution using laser light scattering detection where the average size was reported as 5 µm in whole latex with an enrichment of 2 µm particles in washed latex (Bushman et al. 2006). However, as noted, in this study there were few intact large particles. The values reported by Bushman (2006) may represent coagulated rubber pieces as light scattering cannot discriminate intact particles from coagulated rubber. Light scattering values would be skewed, increasing the reported average size. The observed particles had a diameter size closer to that of Euphorbia lactiflua Phil. (0.208 µm) (Wood and Cornish 2000) or the intermediate sized particles found in the rubber tree (0.25-0.35 µm) (Singh et al. 2003).

High magnification of the particles reveals an uneven particle surface (Figure 4). Surface morphology may be due to impurities sticking to the particle surface as similar surface characteristics are observed on all surfaces including the glass cover slip.
Conclusions

Previous to this study there have been no other reports of microscopic analysis describing the size and structure of rubber particles isolated from prickly lettuce. Prickly lettuce rubber particles are spherical in structure, similar to other rubber particle bearing plants. The particle size distribution was smaller than previously described by other methods, however represent the size of intact particles. Optimization of the isolation method, preventing the coagulation of particles would give a more accurate estimation of the overall size distributions of rubber particles in prickly lettuce.

Acknowledgements

The authors would like to thank the staff, particularly Valerie Lynch-Holm, at the Franceschi Microscopy and Imaging Center, Washington State University for their technical assistance.
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Figure 1. WRPs from prickly lettuce (A, B) and Indian rubber tree (*Ficus elastica*) (B) prior to fixation procedure. Arrow in panel A indicates presence of coagulated rubber. WRPs viewed under light microscope at 40x magnification.
Figure 2. Progressive magnification of fixed, gold coated prickly lettuce WRPs. Viewed using FESEM at 100x (A), 1000x (B), 10,000x (C) and 160,000x (D), magnifications.
Figure 3. Larger sized prickly lettuce rubber particles viewed using FESEM at various magnifications.
Figure 4. Prickly lettuce rubber particles viewed using FESEM at 100,000x magnification.
Chapter 6

Future Directions

Introduction

The heretofore described work on prickly lettuce has led to some interesting discoveries with exciting possibilities for continued research in the study of rubber biosynthesis and general prickly lettuce biology. It is the hope of this scientist that the work carried out will serve as a building block and inspiration for further implementation of ideas and discovery.

Rubber Biosynthesis and Utilization

There is much to be elucidated in the processes involved in rubber synthesis, rubber extraction, and scale-up potential of prickly lettuce as an alternative rubber crop. As a first step in prickly lettuce, a detailed effort in isolating and characterizing the rubber synthesis enzymes present in rubber particles is essential. How many proteins are associated with prickly lettuce rubber particles? More proteins have the potential of leading to allergic responses in end products. Are the particle bound proteins similar to other previously described proteins such as cis-prenyltransferase (CPT), rubber elongation factor (REF) and small rubber particle protein (SRPP)? On a more broad scope, identifying the role played by the isoprene synthesis pathways (MVA or MEP) and the respective enzymes as pertaining to rubber production could be another area of study. The Russian dandelion species *Taraxacum koksaghyz* (Rodin), *Taraxacum brevicorniculatum* (Korol.) and guayule *Parthenium argentatum* (Gray), all members of the compositae family, would serve as an excellent starting point for literature review and experimental comparison. Prickly lettuce as a study tool has advantages over other rubber bearing plant species in that it is diploid, self-pollinating but easily out-crossed, can be
transformed, and although it has a large genome size (2.6 Gb) it’s closest relative, *Lactuca sativa* L., has been sequenced.

Of secondary importance in utilization of prickly lettuce as a crop species would be to find a suitable, non-degradative, and highly efficient method of rubber extraction. As described in chapter four, chemical extraction of freeze dried, milled above-ground material yielded little rubber of low quality. It would be interesting to know what was causing the degradation. Was it oxidation from resin, pigments, or atmospheric oxygen and what could be done to counter the effects such as addition of antioxidants? Alternative extraction timings and methods could also be investigated. Does extraction of fresh material decrease degradation? Could other methods such as floatation or addition of creaming agents be utilized? Does partitioning of plants into organ types change the degradative effects? Efficient extraction methods must be worked out before any cultivar development can occur. Once rubber can be safely removed it will also be important to find ways to utilize the other plant components. Can the cellulosic material be easily converted to ethanol or be used to make other cellulose based products such as paper? Could the resin and sesquiterpene lactones from the latex have some value-added properties increasing the economic feasibility as a crop plant? These are some of the next step questions that need attention.

**Identified QTL and Candidate Genes**

Chapter three describes QTL and potential candidate genes related to rubber production and other interesting prickly lettuce traits. Although the markers used were derived from ESTs, meaning their origin is from a functionally expressed gene, most of them had no known sequence similarity to any annotated genes. The possibility exists to discover what those unknown genes are and how they influence the correlated traits. Of specific interest is marker WSULs-304 and
its relationship to growth phenotypes. Also of interest are markers WSULs-21 and WSULs-374 and their relationship to traits rubber $M_W$ and herbivory. There is a very strong correlation to leaf lobing phenotypes and two markers WSULs-102 and WSULs-212. Unlike the other markers these have sequence similarity to annotated genes from *A. thaliana* and are associated with morphogenesis. As proposed in chapter three, it would be interesting to over express these genes in a non-lobed lettuce background to see if lobing develops or alternatively knock down expression to see if lobing can be abolished. Lobing was clearly a single gene, maybe we landed on it.

**Conclusion**

From the discovery of rubber by ancient Mesoamericans, to the early industrialization and advent of vulcanization in the mid 1800’s, and finally to today’s multi-million tonne harvest, natural rubber has remained a curious substance and a staple of modern society. High demand and supply stability necessitate the discovery of alternative rubber sources. Prickly lettuce is one of the few rubber synthesizing plants able to consistently generate high molecular weight rubber. The research described in this dissertation has outlined new discoveries and problems in prickly lettuce rubber utility. As a newly characterized rubber source, problems are to be expected. Research directed at understanding and optimizing the rubber biosynthesis mechanism, the biochemical stability, and agronomic feasibility will determine if prickly lettuce will become a major player in the rubber industry.
Chapter 7

Philosophical Musings of a Would-Be Scientist

Introduction

What a rare opportunity it is to document in such a location as a doctoral dissertation the current thoughts and opinions pertaining to science, philosophy and life in general of the author. Life is dynamic and ever changing yet holds some evident constants. The accumulation of experiences and additional knowledge leads to new opinions, new philosophies, strengthened convictions and a different view of the world. I would imagine the opinions expressed in the process of writing this chapter will be, to one degree or another, different than what would be conveyed in the years to come. So it is with science. As epiphanies may lead to new life directions, paradigm shifts change what was previously accepted as certainty. In response to the flux of life and science, I will utilize this chapter to convey some of my views on what it means to be a scientist, future positions in academia or industry, reflection on “becoming”, the term impact factor, science and religion, and finish with concluding remarks.

What it Means to be a Scientist

To my delight, I am often enthusiastically introduced by my six year old son as a plant scientist. I do not think he fully grasps the extent of what it means to be a scientist, yet he is aware of what I do and proud that I hold the title. I too am equally excited by the opportunities and challenges inherent to a career in scientific discovery. As a scientist I see myself as a truth seeker, fact finder, a problem solver, and innovator with an innate sense of curiosity.

Let me first attempt to tackle truth and fact. Is there a difference between truth and fact? Fact seems to be more solid and indisputable whereas what is truth can be viewed quite differently between individuals. Differing opinions of truth can be derived from a single or set of
facts. Yet, all variable views of fact originated truths cannot be right. Facts will lead to singular truths. As additional knowledge is acquired, in all realms of understanding, the distinction between truth and fact will become less vague. Truth will become fact. There are scientific facts and scientific truths. As a scientist I seek for truth through the accumulation of facts. The scientific method helps me to experimentally decipher what is fact, indeed how things are. The process of observing and asking questions followed by hypothesis development, experimental design, collection of data, interpretation of results and drawing conclusions will assist in defining what is and what is not. Yet the scientific method is not without its flaws. What appear to be facts must be confirmed. Experiments must be repeated, data collected from multiple sources and checked for consistency. Biases derived from what is or was expected must be set aside. Only through rigorous validation can truths be concluded, applied and built upon. Even then are we seeing reality, or have observer effects influenced outcomes? I believe in the end, when all is made known, there will be no ambiguity between truth and fact.

So why is it that scientific facts and truths are in a constant flux? New discoveries continually augment but sometimes supplant previous ideas. One reason is due to the faults and imperfections of those conducting and reporting science. Human folly is inherent and ever-present no matter how detailed one might be. A decimal misplaced by German chemist Erich von Wolf in 1870 reporting iron content in spinach as 35 mg as opposed to 3.5 mg was compelling enough for studio executive to select the leafy green as Popeye’s supper food of choice (Arbesman 2012). In 1999 two Mars probes were lost due to a few seemingly minor mistakes. The $125-million Mars Climate Orbiter had a misguided trajectory because programmers used English units while NASA navigators used metric units to guide the spacecraft, plunging it into the Mars atmosphere (Stephenson et al. 1999). That same year a signaling problem in the landing
legs during descent of the $185-million Mars Polar Lander initiated pre-mature engine shutdown. One line of missed code could have saved the probe (Casani et al. 2000). These are but a few examples human related scientific errors. History is replete with errors, misled ideas and facts turned false. Continued questioning and verification will roots out old errors, as new ones are made. The goal is to continue moving forward in spite of mistakes.

Another reason for constant scientific amendment is due to a lack of perspective. We don’t and/or cannot see the big picture. I recently attended a department seminar were the speaker, Benildo de los Reyes from the University of Maine, gave a simple analogy on perspective. He shared that he often tells his students of the moose in the window: “If you look out a window what do you see, buildings, trees, people, lots of things. Do you see a moose? No. Does that mean the moose is not there? No, you just cannot see one. By looking out of a confined, defined area ones view or perspective is obstructed.” The complexity of nature forces us to categorize realms of study into separate disciplines further narrowed by subjects then areas of expertise. To advance comprehension in an area of study there must be experts who have vast knowledge on a specific subject. The problem remains however, of the moose in the window. It is beyond our individual human capacity to acquire, combine, and inspect all areas of acquired knowledge and to understand all of the inherent connections. I do feel it is important to always remember the bigger picture, and attempt to make that window a little bigger, or try the view from another window, to gain a new perspective.

Scientists ask questions to solve problems. The problems may be gaps in knowledge, lack of information to make good social decisions or development of more efficient products and technologies. The scientist’s role is to find out why and figure out how. The process requires fabrication of ideas that will take the next step, even if it is in the wrong direction. My few years
of research experience have taught me that scientific discovery is a string of failures dappled with sweet successes. As the German-American rocket scientist Wernher von Braun described it “Research is what I'm doing when I don't know what I'm doing”. The goal is to focus on the question and think of new ways to solve the problem. A scientific innovator must build a foundation of knowledge; learn from previous work without accepting it all as truth, then tap into the limitless potential of human imagination wherein lies the next breakthrough.

I was asked in an interview leading to my first post graduate school employment “what attribute best describes you as a scientist”. My reply was curiosity. Although somewhat cliché, I think in order to be an effective scientist there must be some open or underlying desire to know. During the course of my graduate career curiosity has been a driving force and at times a distracting annoyance. A driving force in that any failures have not left me defeated but rather more resolute to find another way. Distracting because as I learned new techniques or gained knowledge I want to try for myself or take the next step even though my dissertation topic would not directly benefit. I have had to curb my curiosity to some extent to focus and finish projects. A good scientist maintains a healthy amount of insatiable curiosity controlled by a sense of reality and focus.

Academia or Industry

Throughout my graduate career, scientists and non-scientist alike have asked what I plan to do post graduate school, become a professor or go into industry. My reply in most cases has been “I will get a job”, which was the intent of continuing my education. I have never felt so inclined to make an absolution as to where I could land as my graduate career came to a close. It’s always good to plan and set goals but important to be flexible. One never knows what opportunities will present themselves or lack thereof. The rise and fall of economy, budget cuts,
unemployment etc. dictate the available employment options at any point in time. The plan has always been to see what is available at the time of my graduation. Having an open outlook on employment prospects with an ever-present review of my personal satisfaction has and will lead me to where I need to be. As with any defunct situation, if it is not working, make a change. The same method can be made in selection of career path.

There seems to be a general sense that academia is a much more noble pursuit as opposed to selling out in the realm of private industry. At least that is the impression I get from some fellow graduate students and professors. Having now been in the academic arena as a graduate student for over four years I can see the merits and limitations of a career in an academic setting. The ability to direct ones research, to train the next generation of scientists, to be constantly learning and openly adding to the scientific knowledge base are all compelling reasons to pursue an academic career. On the other hand securing competitive research funds, attempting to achieve tenure status, access to limited resources, teaching (if you feel undergrads are morons) detract from the appeal. Academia is a place of learning and training as it was designed to be. My academic career has been a life changing influence not to be matched. Continuing in this path on the next level would be honorable indeed. Yet, no matter how noble an academic career may be, not all, in fact very few, trained students can choose this course.

When I think of industry I often think of the bottom line, a potentially negative aspect of this realm. Projects are decided based on potential earnings and can come and go regardless of scientific value. All companies are in business to make profit, else how would they stay in business? Yet when it comes to influence and impact of scientific discovery as it pertains to applied technologies, especially in agriculture, I feel industry far outweighs any academic endeavor. The developments in industry are meant to be applied on a large scale. Companies
have the resources to invest in advanced technologies that get them ahead of the competition then implement and distribute the technology in turn significantly influencing how things are done. Examples include advances made during the industrial revolution in transportation, electricity, and processing. Medicine would not progress without pharmaceutical company investment. Agriculture could not maintain yields without industry release of pest management compounds, optimized fertilizers, and the next generation of genetically modified crops. Of course one cannot undermine the advances open source academic research has made. Businesses rely on the information generated in these institutions as major flows of knowledge (Adams and Clemmons 2008) yet they are also often the funding sources for that same research. When it is all boiled down, a career in academia or industry are both simply jobs, they will have their positives and negative, their politics, budgets, meetings, layoffs, bonuses etc. Your attitude and work ethic will define success.

**The Prospect of Becoming**

There have been many times in my life where I have questioned my abilities or doubted my potential to be something greater than that of my current state. When I was in elementary school I could hardly imagine myself in high school. After high school I could not see myself as a missionary in a foreign country, before marriage; me, a husband, before children; a father, a graduate student so close to a PhD, unimaginable. Nevertheless here I sit, a high school graduate returned missionary, married with three children writing a dissertation. Really all of those prior-to moments consisted of a lack of vision, a fear of the unknown, a lapse of self-confidence. Fortunately those feelings have not been paralyzing, stifling my progression. I have since learned that “becoming” is a process, that as you do, you become. I need not fear nor undermine my potential; I must merely do, and do my best. Choice is the guiding factor that allows one to
maneuver through the course of life and determine what one is becoming. Interesting that if becoming is used as an adjective rather than a verb it means flattering, appropriate, fitting, right. Becoming is the process of developing into something fitting for each individual.

**Impact Factor**

As a graduate student and budding scientist I have be made more and more aware of the importance given to “impact factor”. Thomas Reuters *Web of Knowledge* has been publishing *Journal Citation Reports* (JCR) since 1975 as a means of determining importance and influence of a journal (Hubbard and McVeigh 2011). The numerical value for each year is determined by taking the number of citations in the current year through the previous two years and dividing by the previous two years (Hubbard and McVeigh 2011). Higher precedence is often given to journals with a high impact factor. Another measure of impact was proposed in 2005 to determine “cumulative impact and relevance of an individual’s scientific research output” called the h-index (Hirsch 2005). The h-index of an individual also relies on citations and takes into account the number of authored papers and how many times they are cited as a whole. If the author has 20 papers and 15 are cited at least 15 times then the h-index is 15. The h-index attempts to give higher relevance to researchers with more papers and more citations over time. A few highly cited papers will not increase ones h-index. These numerical measures, impact factor, h-index and others, are often correlated to the influence and significance of the scientist’s contributions to the scientific community. The use of such evaluation systems has utility but also drawbacks. In 2009 a seemingly obscure journal *Acta Crystallographica – Section A* made a 20 fold jump to the second highest impact factor position due to a massively cited paper “A short history of SHELX” (Sheldrick 2007) which outlines the development of an open source software program used in crystal structure determination (Schwarzenbach et al. 2010). At face value it
would appear that this journal and author have made a spectacular achievement. It could also be argued that the impact factor measure is completely flawed. In reality the single paper presents nothing ground breaking but rather a point of reference for many fields using the software. Previous to the publication the unpublished software was cited as individual programs themselves (Schwarzenbach et al. 2010). The journal will enjoy two years of high ranking based on the impact factor assessment method then return to the previous standing of 2.0-2.5, representing an anomaly not necessarily a flaw. Although there is no perfect metric to determine ones scientific influence the use of such is helpful for assessment and comparison purposes as long as they are not the sole means evaluation. Publishing in journals within a specific field is more important than just shooting for the highest impact factor. I personally never expect to publish in the highest rated journal CA-A Cancer Journal for Clinicians, the field will never apply to me. It is important to take into account other activities other than publication and citation number as well. Publication in non-journal settings such as agricultural bulletins can have a far reaching impact to the farmer. Teaching, mentoring and other outreach activities can inspire the next generation of scientists, with implications that could far outreach any h-index.

When I think of impact factor, I think of the impact my being in the world will have. A successful career is part of that impact but not the ultimate definer of my influence. Impact, when thought of in this way, is much more of a personal all encompassing assessment of one’s life impression. I already know my life has influenced others as others have touched mine. It is then a question of what kind of impact one will have. Hopefully, the impact will be a positive one. My wife will know of my love for her, and always will be grateful for our partnership. My children will grow with confidence in themselves with a knowledge there father is on their side, and they in turn will have a positive impact. My friends and associates can find in me trust,
loyalty, and a willingness to serve. This impact factor will be a self assessment and a contact assessment. You can judge the greatness of an individual by the influence they have had in the lives of those around them. In these regards my impact factor far outweighs any assessment Thomas Reuters can devise.

**Science and Religion**

The final merge between fact, truth, and faith, science and religion. Where shall I take my stance? That has long been the question through my adolescence and adulthood, a question that all people must face. My beliefs have been shaped by the teachings in my home which were centered in Christianity and conveyed through membership in The Church of Jesus Christ of Latter-Day Saints (LDS). Experience, study, and search for truth have further solidified my faith and testimony that there exists a living God who has made a plan with purpose to human life.

Review of some of the most notable and read science philosophers would mildly discount or outright contradict my stance. A 1969 interview with Karl Popper sealed until after his death revealed an agnostic view on Gods existence with religious undertone of belief: “When I look at what I call the gift of life, I feel a gratitude which is in tune with some religious ideas of God. However, the moment I even speak of it, I am embarrassed that I may do something wrong to God in talking about God.” (Zerin 1998). Philosopher Alfred Ayer rejected atheism and agnosticism as both take the position of Gods existence as a meaningful hypothesis, which he did not believe (Ayer 1952). It is no surprise to me that most science philosophers cannot accept deity or forms of religion.

Although the trend of disbelief continues throughout the world, there are scientists and philosophers who see the value and necessity in taking a religious position as complementary to their work. Of science and religion, theoretical chemist Henry Eyring was of the opinion there is
no distinction “Is there any conflict between science and religion? There is no conflict in the mind of God, but often there is conflict in the minds of men.” (Eyring 1983). Eyring felt science and religion will converge as pure truth is found. Even Albert Einstein an open agnostic viewed the scientific pursuit of truth and understanding stemmed from a religious sphere were faith in a rational, explicable world must be exercised: “Science without religion is lame, religion without science is blind.” (Science, Philosophy, and Religion: A Symposium 1941). There must be some link between the search for understanding of the physical world and the spiritual.

Are my emotions, desires, and feelings, solely an artifact of heredity; an evolutionary accumulation of biological successes leading to a cerebral self-fulfilling biochemical soup? To a certain extent, yes. I cannot help but see linkages between my biological self and that of my ancestry and posterity. Yet, am I left to the fortune or folly of genetic recombination and mutation. I may have or be predisposed to certain disease and bodily dysfunction as determined by me heredity, but my genetic make-up does not determine my thoughts and in turn my actions. I am not so biologically preprogrammed that predilections cannot be changed or my character concluded. My being goes beyond that of a chemical concoction. There is an eternal nature imbued within my mortal frame that has given me life and will continue once the biochemistry reaches equilibrium. Of this truth I cannot scientifically prove, so far. Maybe the actuality that it cannot be shown or proven is a part of the design. It must be reasoned and felt. Many of the most important life lessons cannot be scientifically manifest and can only be learned through emotions, desires, and feelings. Perhaps the decision as to the divine must be made solely on and anchored in faith. When it comes to religion I don’t see a conflict, only a choice. All major choices I have ever made have been laid out in logic but made in emotion. Belief is always a choice whether the sought truth is scientific or religious in nature.
It seems somewhat absurd to reason that the presence and existence of life and truly my own cognition is a happenstance of millions, correction, billions of years of luck. Is the creation of our world and the apparent evolution of innumerable creatures a fortunate event never before or hereto after achieved? It appears so as we have yet to confirm life outside our own sphere, but I think not. Scripture has stated “worlds without number have I created” (Pearl of Great Price: Selections from the Book of Moses 2013) life has been created elsewhere. There is too much of complex structure to conclude all these things have happened by accident. I propose that there are fundamental, universal laws that govern the nature of the universe on all levels. From the apparent structure of the observable universe itself (Springel, Frenk, and White 2006), to the sub-atomic (Amsler et al. 2008), evidence of composition and structure emerge. Each new validated discovery leads us ever closer to the fundamental, however complex. The existence of a divine organizer, formulator, and designer is not beyond reason.

Concluding Remarks

So what does all of this mean? As my chosen profession, I am a scientist. I am doing my best to discover fact filled truths that will have a positive impact on the world. My impact will hopefully extend farther than my professional contributions. With every life challenge and experience I chose to become something greater than my previous self. As my defense date nears and the summation of five years of work is evaluated I know that the final outcome is not just the scientific contribution embodied in the work but also a change of the individual performing the work. My hope in becoming a Ph.D. recipient is that it will have played positive role in the ultimate assessment of who I am and will be in this life and throughout eternity “…the Final Judgment is not just an evaluation of a sum total of good and evil acts—what we have done. It is
an acknowledgment of the final effect of our acts and thoughts—what we have *become*” (Oaks 2000).
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Appendix A

Preface

The following appendix contains material performed and published by the dissertation author. Although the study topic does not pertain to the previously described work on prickly lettuce rubber production, it contains a significant contribution by the author in fulfillment of said degree. The work was published in Pest Management Science and is formatted according to the journal requirements.

Uptake, Translocation and Metabolism of Aminocyclopyrachlor in Prickly Lettuce, Rush Skeletonweed, and Yellow Starthistle


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Abstract

BACKGROUND: Aminocyclopyrachlor is a new herbicide proposed to control broadleaf weeds and shrubs in non-crop and rangeland systems. To better understand observed field efficacy, uptake and translocation of foliar applied aminocyclopyrachlor (DPX-MAT28) and aminocyclopyrachlor methyl ester (DPX-KJM44) were evaluated in two annuals, prickly lettuce (Lactuca serriola L.) and yellow starthistle (Centaurea solstitialis L.) and one perennial, rush skeletonweed (Chondrilla juncea L.).

RESULTS: Absorption and translocation varied between species. While absorption of DPX-KJM44 was greater than absorption of DPX-MAT28, rush skeletonweed absorbed the most followed by yellow starthistle and prickly lettuce. Overall total translocation of either herbicide was highest in yellow starthistle followed by rush skeletonweed and prickly lettuce. Proportional herbicide movement between species was similar with the majority translocating to developing shoots. However, in rush skeletonweed, early translocation was directed to root tissue. In rush skeletonweed, no DPX-MAT28 metabolism occurred while DPX-KJM44 was rapidly de-esterified and translocated as DPX-MAT28.

CONCLUSION: Aminocyclopyrachlor absorption and translocation is dependent on active ingredient structure and species sensitivity. Highly sensitive species like prickly lettuce absorb and translocate less material than relatively less sensitive species like rush skeletonweed. De-esterification of DPX-KJM44 appears to delay translocation of the resulting acid in yellow starthistle and rush skeletonweed.

Keywords: Absorption, autoradiography, auxinic herbicides, growth regulator, pyrimidine carboxylic acid, translocation.
1 Introduction

Aminocyclopyrachlor is a new pyrimidine-based auxin type herbicide proposed to control broadleaf weeds and shrubs in noncropland systems including roadsides, industrial areas, and rangeland. Aminocyclopyrachlor (DPX-MAT28) (Figure 1-1) and aminocyclopyrachlor methyl ester (DPX-KJM44) (Figure 1-2) were the active ingredients available for both laboratory and field studies. Control of forbs has been observed in several dicot families including Asteraceae, Fabaceae, Chenopodiaceae, Convolvulaceae, Solanaceae and Euphorbiaceae and woody plant species such as red maple (Acer rubrum L.), box elder (Acer negundo L.), common hackberry (Celtis occidentalis L.), white willow (Salix alba L.), blackgum (Nyssa sylvatica Marsh.), mesquite [Prosopis juliflora (Sw.) DC.] and American elm (Ulmus Americana L.).\textsuperscript{1-4} While effective on broadleaf weeds and shrubs, aminocyclopyrachlor also has reduced activity on some monocot species. Tolerance by monocot species is an important quality for restoration of desirable native perennial grasses in grasslands and broadleaf weed control in established turf.\textsuperscript{5-7} Other unique features of aminocyclopyrachlor are low proposed use rates, low mammalian toxicity, and favorable environmental profile.\textsuperscript{4-5}

Aminocyclopyrachlor is structurally similar to other pyridine-based herbicides such as picloram, clopyralid and aminopyralid.\textsuperscript{8} Aminocyclopyrachlor has an additional nitrogen atom in the heterocyclic ring, making it the first pyrimidine-based growth regulator herbicide (Figure 1). The specific mode of action has yet to be established but general consensus is that it behaves similarly to auxinic herbicides with symptoms including stem and leaf epinasty and wilting.\textsuperscript{8}

The two compounds are almost identical in structure; DPX-KJM44 adds a methyl group to the carboxyl end. In comparison to DPX-MAT28, DPX-KJM44 is more lipophilic. The octanol/water partition coefficient ($K_{ow}$) is 1.87 (at 20°C) for the DPX-KJM44 and -2.48 for the
DPX-MAT28 (pH 7 at 20°C). Methyl ester herbicide derivatives are commonly used to increase absorption as they facilitate penetration through the lipophilic leaf cuticle. Upon application, esterase action in the cuticle and the apoplast actively remove the ester, releasing the free acid into the plant cells and into the phloem by ion trapping. Rapid de-esterification produces a strong concentration gradient across the cuticle promoting continuous uptake of the herbicide, increasing total absorption. The Kleier model of xenobiotic transport predicts relatively low potential for phloem transport for compounds with a low $K_{ow}$ like the free acid of aminocyclopyrachlor, even though it has a near optimal $pKa$ (4.65).

Three weed species, all members of the Asteraceae family, were chosen as model plants to understand absorption, translocation, and metabolism of DPX-MAT28 and DPX-KJM44. The three species were selected due to observed differences in symptomology and control, but they are also widely distributed and troublesome weeds throughout the Pacific Northwest and the western United States. Prickly lettuce is an annual herbaceous weed, native to the Mediterranean region. It is found throughout North America and Hawaii. Prickly lettuce is a troublesome weed in cropping systems in the Pacific Northwest that can cause up to 80% yield loss in soybean in Canada if not controlled. Biotypes of prickly lettuce have developed resistance to several classes of herbicides including acetolactate synthase inhibitor herbicides and more recently 2,4-D.

Rush skeletonweed is an herbaceous perennial with an extensive taproot system that can reach over two m in length. The species is an obligate apomictic species that also reproduces through adventitious buds on roots. Both seed and vegetative propagules are produced. Seed are apomictic, and mature plants generating 1500 flower heads and 20,000 seeds in a single season. Vegetative re-growth occurs through rootstocks initiated from the base of previous
years’ growth and bud expansion from lateral roots. Upon fragmentation, shoots can develop from as little as one centimeter of a root section.\textsuperscript{21} For effective chemical control of rush skeletonweed, absorbed herbicide must translocate to the root system. Effective control of rush skeletonweed can be achieved with mixtures of clopyralid, 2,4-D, MCPA, or dicamba applied over three consecutive seasons.\textsuperscript{23}

Yellow starthistle is an annual herbaceous weed from the Mediterranean region and may have been introduced to California from Spain in contaminated alfalfa seed in the early to mid-1800’s.\textsuperscript{24} Yellow starthistle infests approximately 6 million hectares in California alone with substantial populations in Oregon, Washington and Idaho.\textsuperscript{25} Several strategies for control of yellow starthistle in California have been developed including grazing\textsuperscript{26}, mowing\textsuperscript{27}, large-area burns\textsuperscript{28}, competitive planting of grasses and clovers\textsuperscript{29}; or biological control.\textsuperscript{30} Of these, prescribed burning in combination with applications of clopyralid has been most effective for control method for yellow starthistle infestations.

Field application rates of aminocyclopyrachlor vary for control of each of these species. Prickly lettuce is highly sensitive at low use rates and control can be achieved 70 g ai ha\textsuperscript{-1} (C. William Kral, personal communication). In contrast, dosage and timing of application affect the long term control of rush skeletonweed. Applications of DPX-KJM44 to spring and fall rosettes at rates of 70, 140 and 210 g ai ha\textsuperscript{-1} give > 90\% control.\textsuperscript{31} Applications at the floral bud stage are less effective at lower rates yielding 62, 81 and 91\% control at each rate, respectively. Further evaluation of rosette density found that although initial control was achieved in the fall rosette application at all rates, rosettes formed with the 70 g ai/ha application suggesting insufficient systemic control.\textsuperscript{31} Effective control of yellow starthistle with aminocyclopyrachlor is possible at application rates between application rates needed for rush skeletonweed or prickly lettuce.
Effective control of weed species is affected by initial absorption and subsequent translocation of sufficient herbicide to the site of action to be phytotoxic. As aminocyclopyrachlor is a new class of auxinic herbicide, limited studies have been conducted to determine absorption, translocation, and metabolism of the compound in targeted plant species. On a fundamental level, structural differences between the two active ingredient forms may affect total absorption and translocation and could be dependent on the rate of de-esterification. The objective of these studies were to (1) compare the absorption and translocation of DPX-MAT28 versus DPX-KJM44 in prickly lettuce, yellow starthistle, and rush skeletonweed; (2) determine the rate of conversion of the DPX-KJM44 to DPX-MAT28, and (3) determine whether metabolism of DPX-MAT28 in rush skeletonweed occurs.

2 Materials and Methods

2.1 Plant Material

Absorption and translocation studies were conducted on prickly lettuce, rush skeletonweed, and yellow starthistle. Rush skeletonweed plants were grown from root fragments with fully formed rosettes. Plant and root fragment were separated from the mother plant and placed into 10 cm square plastic pots containing a 3:1 mixture sand:potting media (LC1 Mix. Sun Gro Horticulture Distribution Inc., Bellevue, WA). Rosettes developed from the transplanted rhizome fragments as they would in the field during the fall or spring. Plants were given 2 weeks to recover from transplant stress before treatment. Prickly lettuce and yellow starthistle were grown from seed. Prickly lettuce seeds were collected in southeastern Washington. Yellow starthistle seeds were collected from a site near Lacrosse, Washington. Seeds of both species were planted into 10 cm square plastic pots containing potting media. Following emergence, plants were thinned to one per pot and transplanted into 3:1 sand:potting media. Plants were
grown in a greenhouse and kept at of 26 °C daytime and 22 °C nighttime (± 3 °C) temperatures. Natural light was supplemented with sodium vapor lighting to give a 14 h photoperiod that coincided with the daytime portion of the temperature profile. Plants were subirrigated as needed. Seeded plants were grown for 4 weeks to a 4 to 5 leaf rosette stage.

Metabolism of aminocyclopyrachlor was investigated in rush skeletonweed. Rush skeletonweed plants were grown from 5 cm root fragments placed into 16 cm round plastic pots containing a soil mixture of 3:1 sand:potting media. Pots were placed in a greenhouse under the same conditions as previously described; however, more time was required for plants to develop than in the absorption and translocation study. Plantlets emerged from root fragment nodes and were treated when the resulting rosette had 8 to 10 leaves, approximately 2 mo.

2.2 Absorption and Translocation of DPX-MAT28 and DPX-KJM44

Plants at the 4 to 5 leaf stage were selected for treatment. A 1 cm² portion of the adaxial side of the youngest fully expanded leaf was marked and covered to prevent spray solution from contacting that portion of the leaf. Plants were treated with a non-radiolabeled mixture of herbicide containing 210 g ai ha⁻¹ of either DPX-MAT28 or DPX-KJM44 and a nonionic surfactant (NIS) (Induce® nonionic surfactant, Helena Chemical Company, Memphis, TN) at 0.25% v/v using a carrier volume of 300 L ha⁻¹. Immediately after herbicide application, 5, 0.5-µL droplets of technical grade DPX-MAT28 (specific activity 350.3kBq µmol⁻¹) or DPX-KJM44 (specific activity 375.0 kBq µmol⁻¹) [pyrimidyl 2-¹⁴C] was applied to the marked leaf area, avoiding the leaf midrib. Aminocyclopyrachlor spotting material was dissolved in 200 µL MeOH, diluted with deionized water, and 0.2% NIS (v/v). The amount of radioactivity applied was approximately 3.33 kBq. A 25 µL glass syringe (Hamilton Company, Reno, NV) placed in a
repeating dispenser (PB600 Dispenser, Hamilton Company, Reno, NV) was used for applications.

Plants were harvested at 2, 4, 8, 24, or 72 HAT and divided into seven parts, leaves above the treated leaf, leaves below the treated leaf, crown, root, the treated portion of the treated leaf, the acropetal portion of the treated leaf, and the basal portion of the treated leaf. The treated portion of the treated leaf was washed by swirling the plant part in a scintillation vial containing 1 mL of methanol:deionized water (1:1 v/v) and 1% (v/v) NIS to wash off nonabsorbed radioactive material for 20 s. To the rinsate, 20 mL of scintillation fluid (EcoLume, MP Biomedicals, Solon, OH) was added prior to quantification via liquid scintillation spectrometry (LSS) (Tri-Carb 2900TR, PerkinElmer, Shelton, CT). Each plant part was placed into a paper envelope and dried at 40 °C for 48 h. Dried portions were weighed and then combusted using a biological oxidizer (Biological Oxidizer OX500, R.J. Harvey Instrument Corporation, Tappan, NY). The resulting $^{14}$CO$_2$ was captured into 15 mL scintillation cocktail (R.J. Harvey Instruments, Tappan, NY) at a combustion time of 2 min per sample. Radioactivity in oxidized samples was quantified by LSS.

The absorption and translocation studies were organized as a split-split plot with herbicide treatment as the main plot, time after treatment as the sub-plot, and plant parts as the sub-sub-plot. Treatments were replicated four times, and the experiments were conducted twice. Absorption was determined by subtracting the amount of $^{14}$C recovered in the leaf wash from the total amount of radioactivity recovered from all plant parts (amount applied). Translocation out of the treated leaf was calculated as percent applied by summing of radioactivity recovered in all plant parts excluding the treated leaf and dividing by the total amount of absorbed radioactivity.

2.3 Metabolism of DPX-MAT28 and DPX-KJM44
The metabolism study was similar to the absorption and translocation study with a few exceptions. Rush skeletonweed plants were prepared and treated as previously described for absorption and translocation. At harvest, the entire spotted leaf was kept intact. To ensure detection of radiolabeled material, the amount of activity applied to each plant was increased to 6.00 kBq. At harvest, individual plant parts were wrapped in aluminum foil, sealed in a 7.6 by 17.8 cm plastic bag, and stored at -20°C until extraction.

For extraction, frozen plant parts were weighed then placed into a conical centrifuge tube. Depending on the size of the part, 15 mL (VWR International Inc., West Chester, PA) or 50 mL (Evergreen Scientific Inc., Los Angeles, CA) centrifuge tubes were used. All extraction and dilution steps were conducted in methanol. Methanol was added to each tube, 3 mL for the 15 mL tube or 10 mL for the 50 mL tube, and the plant tissue was homogenized (Polytron, Brinkmann Instruments. Westbury, NY). The homogenizer was rinsed with 5 mL methanol into the tube. Tubes were then centrifuged (Beckman GS-6R Centrifuge, Beckman Instruments Inc., Palo Alto, CA) at 2000 g for 10 min and the supernatant decanted into a second centrifuge tube. Homogenization and centrifugation were carried out three times for each plant part. Following the third centrifugation, the pellet was air dried, wrapped in aluminum foil and oxidized in biological oxidizer as previously described to determine the efficiency of the extraction. The average efficiency of extraction was 85.4% for DPX-MAT28 and 93.2% for DPX-KJM44. Combined extracts were dried under a stream of air at room temperature and then re-eluted in 500 µL methanol. To evaluate potential post-extraction degradation, 5 µl radiolabeled herbicide solution was added to extracts from nontreated plants extracted as described above.

Separation of herbicide metabolites was done by normal-phase thin layer chromatography (TLC). Silica gel plates (20 x 20 cm) (Whatman®, Partisil K6 Absorption TLC plates with
preadsorbant zone, Florham Park, NJ) were used as stationary phase and a solvent system of methanol, isopropanol, ethyl acetate, and acetic acid (7:1:1:1, v/v) as the mobile phase. Plates were pre-scored into nineteen individual 1 cm lanes and a 250 µL aliquot was applied 3 cm above the bottom of the plate, leaving a blank lane between each replicate. The area of application was maintained to ~5 mm by successive spotting and drying. As standards, 0.5 µL stock solutions of \(^{14}\text{C}\) DPX-MAT28 and \(^{14}\text{C}\) DPX-KJM44 (1.2 kBq), dissolved in methanol, were applied on the center lanes of the plate. Plates were developed for 75 min at which point the mobile phase had moved 155 cm above the origin then allowed to air dry. The radioactive positions, proportions and corresponding relative mobility (R\(_f\)) of \(^{14}\text{C}\) parent compounds and metabolites were determined by scanning TLC plates with a AR-2000 radiochromatogram scanner (AR-2000 radio-TLC Imaging Scanner, Bioscan Inc., Washington, DC). Radioactive trace peaks were manually integrated. Peaks below 1% of total radioactivity were rejected. Parent herbicide was identified by comparing R\(_f\) values from the corresponding standard. Data consisted of the percentage parent herbicide, the sum percentage of all metabolites that were more polar than the parent herbicide, and the sum percentage of all metabolites that were less polar than the parent herbicide.

2.4 Autoradiography

To better visualize the final position of the radiolabeled herbicide, treated plants were exposed on x-ray film. A portion of the second fully expanded leaf of each weed species was marked and treated with 3.33 kBq of radioactive herbicide solution as described in the absorption and translocation experiment. Entire plants were harvested at 24 and 72 HAT. Soil was removed from the roots by washing with distilled water. Plants were immediately mounted between two layers of 22 × 28 cm\(^2\) newspaper. Curled leaves were spread carefully to avoid the treated leaf.
contacting other plant parts and pressed in wooden press boards. The stacking order in the press from bottom to top was wooden plate, cardboard, newspaper, fully spread plant, newspaper, cardboard and second wooden plate. The press was held together by large metal binder clips on all four sides and stored at -20 C. After one week, plants were taken out of the press and wrapped in one layer of plastic film, again avoiding contact of the treated leaf with other plant parts. Pressed whole plant materials were placed in a 35 x 43 cm cassette and a sheet of x-ray film (Kodak Scientific X-OMAT Blue Full Speed MXB/CSB1 imaging film, Perkin Elmer, Inc., Waltham, MA) positioned over the pressed plant. Exposure times were 4 weeks at -20ºC. After exposure, the imaging films were developed. The film was dried for half an hour and scanned with a flatbed scanner. Non-treated plants were used as a control.

2.5 Statistical Analysis

The five plant portions of quantified radioactivity were combined as the total absorbed into the plant (the sum of the radioactivity from the treated leaf, above treated leaf, below treated leaf, and root portions), the total radioactivity translocated out of the treated leaf (the sum of the radioactivity from the above treated leaf, below treated leaf, and root portions), the above treated leaf portion, and the below treated leaf portions (the sum of the radioactivity from the below treated leaf and root portions). Data were subjected to ANOVA using SAS 9.2 that employed PROC MIXED\textsuperscript{32}. ANOVA indicated that trial effects for absorption, total translocation out of the treated leaf, and translocation to the root were not significant for any plant species or harvest interval (P > 0.05), therefore data were pooled over trials. Non-linear regression was conducted on both absorption and translocation replicate data to describe overall treatment patterns. Absorption data was fit to an asymptotic, exponential maximum two term model as the following equation:
\[ y = a^*\left[1 - \exp(-b^*x)\right] \]  

where \( y \) is the percent absorption expressed as the percent of the applied dose; \( a \) is the upper asymptote, or theoretical absorption maximum; \( x \) is time, expressed as hours after treatment; \( b \) is a slope term that has a positive relationship to the rate with which absorption reaches maximal levels. Identical regression analysis has been used to model absorption of imazamox in feral rye to understand MCPA-ester/imazamox synergism.\(^{33}\)

A three term Gompertz equation was used to relate translocation over time. The Gompertz equation was:

\[ y = a^*\left[ \exp\left(-\exp\left(-\frac{x - k}{b}\right)\right)\right] \]  

where \( y \) is the percent translocation expressed as the percent of the applied dose, \( a \) is the asymptote or the maximum translocation expressed as the percent applied, \( x \) is time, expressed as hours after treatment; \( k \) is the point of inflection (in h) and \( b \) is the slope of the curve at the point of greatest inflection.

Coefficients of determination \((R^2)\) were calculated for all regressions. For the Gompertz and exponential maximum equations fitted to the data, an approximate \(R^2\) value was obtained by subtracting the ratio of residual sums of squares to corrected total sums of squares from 1.\(^{34}\) The \(R^2\) and residual mean squares were used to determine goodness of fit to nonlinear models.

For the metabolism study, data consisted of area normalized percentage of either DPX-MAT28 or DPX-KJM44. Statistical procedures were similar to the absorption and translocation study and percentage metabolism was modeled using the equation:

\[ C = y0 + C_0 e^{-kt} \]  

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where \( y_0 \) is the point where the decay rate equals 0; \( C_0 \) is the percent in the treated leaf at time zero minus \( y_0 \); \( k \) is the first-order rate constant (h\(^{-1}\)); and \( t \) is time (h). Aminocyclopyrachlor half-life (\( T_{1/2} \)) was calculated from equation [4]:

\[
T_{1/2} = \frac{\ln 2}{k}
\]

where \( k \) is the first order rate constant calculated in equation [3].

3 Results

3.1 Absorption and Translocation of DPX-MAT28 and DPX-KJM44

Absorption of applied DPX-MAT28 was biphasic, and the majority of DPX-MAT28 was absorbed within 24 HAT by each species (Figure 2B). In prickly lettuce, absorption of DPX-MAT28 increased only slightly as harvest interval increased, but never exceeded 5% of applied radioactivity. Increased absorption of DPX-KJM44 was observed in prickly lettuce in the first 24 HAT (9.9% of the applied) (Figure 2A). A maximum of 10.3% of applied radioactivity was absorbed by prickly lettuce at 72 HAT. Based on observed injury, absorption rates in prickly lettuce appear low due to rapid herbicidal activity – symptoms were observed within 24 h of treatment and epinasty in prickly lettuce was much more pronounced than in the other two species.

In yellow starthistle, absorption was 10.6% of the applied DPX-MAT28 in the first 24 h, and was 14.6% of the applied DPX-MAT28 at 72 HAT. Absorption of DPX-KJM44 into yellow starthistle increased for the duration of the experiment, reaching 31.6% of the applied DPX-KJM44. Absorption of aminocyclopyrachlor by rush skeletonweed was greater than the other two species, with 64.1% of the applied DPX-MAT28 and 53.2% of DPX-KJM44 absorbed by 24 HAT. Absorption in rush skeletonweed at 72 HAT was similar to that of the 24 HAT observations for each compound (Figure 2).
Translocation out of the treated leaf to the rest of the plant was greater in yellow starthistle than the other two species (Figure 3). At 72 HAT, yellow starthistle translocated 19.3% of the applied DPX-KJM44 out of the treated leaf. Rush skeletonweed and prickly lettuce translocated less, 6.5% and 3.2% of the applied radioactivity respectively. Translocation of DPX-MAT28 out of the treated leaf was lower than DPX-KJM44 for each plant species. At 72 HAT, 1%, 3.6%, and 10.7% of the applied DPX-MAT28 had translocated out of the treated leaves of prickly lettuce, rush skeletonweed, and yellow starthistle, respectively. In rush skeletonweed, translocation out of the treated leaf was greater at the 24 HAT harvest than the other harvest intervals (8.7% of the applied).

In yellow starthistle, translocation of radioactivity was directed toward the above ground portions (all plant parts excluding roots) of the plant consisting of the crown and upper leaves (Figure 4). Translocation to the above ground portion of yellow starthistle was similar regardless of active ingredient form, and was > 9% at 72 HAT. Translocation of radioactivity to the above ground portions in prickly lettuce and rush skeletonweed was less than yellow starthistle, and was less than 2.3% of the applied for both compounds. Although less than 2.3% of the applied radioactivity translocated to the above ground portions in rush skeletonweed, 3.6% of the applied radioactivity moved to the below ground portions of the plant at 72 HAT when the radioactivity was applied as DPX-KJM44 (Figure 5). When applied as DPX-MAT28, movement of radioactivity to the below ground portion in rush skeletonweed was also 3.6% at 24 HAT, although by 72 HAT radioactivity was lower (1.3% of the applied) and similar to that of yellow starthistle.

3.2 Metabolism
No metabolites of aminocyclopyrachlor were observed. All radioactivity recovered from rush skeletonweed was DPX-MAT28 (Rf=0.85) or DPX-KJM44 (Rf=0.93) (Figure 6). No DPX-KJM44 was recovered from outside the treated leaf, indicating that DPX-KJM44 was de-esterified and translocated as DPX-MAT28. De-esterification was biphasic and occurred rapidly in the first 24 HAT but slowed thereafter (Figure 7). At 72 HAT, 17.9% of DPX-KJM44 remained in the treated leaves. Based on first-order kinetics, the half-life of the DPX-KJM44 in rush skeletonweed was 3.5 h.

3.3 Autoradiography

Autoradiographs of the treated plants were consistent with the empirical data collected through biological oxidation of the plant samples (Figure 8). Prickly lettuce and yellow starthistle accumulated radiolabeled material in the newer developing leaves, indicating acropetal movement of the radiolabel. Rush skeletonweed autoradiographs had a higher quantity of radioactivity, judged by the relative intensity of the radioactivity, localized in the root tissue, followed by accumulation in new leaves. Movement of radiolabel in rush skeletonweed was basipetal. Although not quantitative, autoradiographs provide validation of the absorption and translocation studies and allow visualization of localization of the herbicide in the various plant tissues. The images also suggest DPX-MAT28 is both phloem and xylem mobile.

4 Discussion

The objective of these studies were to compare the absorption and translocation of DPX-MAT28 to DPX-KJM44 in prickly lettuce, yellow starthistle, and rush skeletonweed as the three species differ in their response to a field use rate of the two forms of the herbicide. Species sensitivity and herbicide structure affects both absorption and translocation of the active ingredient, aminocyclopyrachlor. When treated with DPX-MAT28 or DPX-KJM44, the most
sensitive species, prickly lettuce, absorbed the least radioactivity, regardless of herbicide form. Yellow starthistle absorbed more DPX-MAT28 and DPX-KJM44, and the least sensitive weed, rush skeletonweed, absorbed the most DPX-MAT28 or DPX-KJM44. Yellow starthistle and prickly lettuce absorbed more DPX-KJM44 than DPX-MAT28. Ester herbicide derivatives enhance absorption by increasing the lipophilic nature of the compound, facilitating cuticle penetration.10-12

Conversely, radioactivity translocated in greater quantities out of the treated leaf when applied as DPX-MAT28 than when applied as DPX-KJM44 at the 2 and 4 h harvest intervals in rush skeletonweed and yellow starthistle. Ester herbicide derivatives are not very mobile in the symplast due to their lipophilic nature. Although permeable to the lipophilic wax layers of the leaf cuticle, methyl ester herbicide derivatives have reduced permeability to the hydrophilic pectin and cellulose layers of the cuticle and apoplast. Esterase activity in the cuticle and apoplast create a more hydrophilic free acid that can then reach the plasma membrane and symplast.13-15. The de-esterification reaction required to convert the DPX-KJM44 into the more mobile and herbicidally active DPX-MAT28 likely slowed the availability of DPX-MAT28 for translocation. As a consequence of the increased translocation of DPX-MAT28 at the early harvest intervals, the deregulation of sink strength likely occurred more rapidly in DPX-MAT28-treated plants and contributed to the lower overall total absorption and translocation observed with that compound form. In later harvests, greater amounts of radioactivity translocated when applied as DPX-KJM44 than when applied as DPX-MAT28 as more DPX-KJM44 was absorbed at those later harvests. Growth inhibition is one of the several auxin-herbicide-mediated responses in plants, and usually occurs within 24 h. Growth inhibition and deregulation of sink strength occurred more rapidly in prickly lettuce or when aminocyclopyrachlor was applied as
DPX-MAT28. The result of the deregulation of growth in prickly lettuce and DPX-MAT28 applications was the lower observed absorption compared to yellow starthistle or rush skeletonweed in this study or absorption by Canada thistle\textsuperscript{9} or the DPX-KJM44 derivative, respectively.

In addition to esterase activity and de-regulation of sink strength, difference in herbicide perception may account for the varying degrees of response observed in this experiment. When exposed to high auxin levels or persistent auxin xenobiotics, such as non-metabolized auxinic herbicides, plants elicit a metabolic and physiological auxin response that begins with TIR1/AFB auxin receptors.\textsuperscript{35} The presence of auxins causes release and degradation transcriptional repressors (AUX/IAA), which in turn leads to derepression of auxin response factors (ARFs) allowing for transcription induced activation of auxin-reactive genes.\textsuperscript{35} Initial responses, once perception occurs, include upregulation in ACC synthase leading to elevated ethylene levels, this in turn activates ion channels and +H-ATPases causing abnormal cell elongation and epinasty.\textsuperscript{36} Within hours of perception, abscisic acid (ABA) biosynthesis genes are over expressed causing stomatal closure, meristematic growth inhibition, and senescence. The further production of reactive oxygen species, and cyanide, leads to membrane degradation, chloroplast damage, and vasculature failure. The summation of metabolic and physiologic response causes plant death.\textsuperscript{36} Rush skeletonweed auxin perception to aminocyclopyrachlor may be less significant than that observed in the other weed species tested. Indeed, mutations in TIR1 homologue AFB5 from Arabidopsis conferred resistance to picloram with no cross resistance to 2,4-D or IAA\textsuperscript{37} suggesting chemical selectivity and substrate specificity to auxin receptor proteins. Unperceived or reduced sensitivity to auxinic herbicide presence would allow for
continued absorption and translocation of herbicide as the biochemical lethal cascade of events would take longer to complete.

The de-esterification rate of DPX-KJM44 was slower in rush skeletonweed than Canada thistle. At 72 HAT, 18% of the applied DPX-KJM44 remained as DPX-KJM44 in rush skeletonweed. More rapid de-esterification was observed in Canada thistle - 18% of DPX-KJM44 remained at 6 HAT, and the half-life in Canada thistle appeared to be less than 1 h. Indeed, the de-esterification rate in rush skeletonweed was slower than many other ester herbicide derivative auxin and growth-regulator type herbicides.

Metabolism profiles have been observed in other auxinic herbicides such as aminopyralid and clopyralid. De-esterification rates of various herbicides have been observed to differ between species. Differences in the rates of de-esterification are most likely related to the presence of plant esterases and their affinity to herbicide substrates. Indeed, Gershater reported that the auxinic herbicide 2,4-D methyl was the preferred substrate when compared to other agrochemical pro-herbicide esters, namely fenoxaprop-ethyl, fenthionprop-ethyl, clodinafop-propargyl and bromoxynil-octanoate. When compared between species the rate of 2,4-D-methyl carboxylester hydrolysis ranged from 365.2 ± 54.8 pkat/mg extracted protein in Arabidopsis thaliana (L.) Heynh. to 54.0 ± 3.7 pkat/mg in barnyardgrass (Echinochloa crus-galli L. Beauv), indicating species dependent differences in carboxylesterase catalytic activity. Comparing the carboxylester hydrolysis rates in Canada thistle and rush skeletonweed would be of interest, as the rate of de-esterification could affect control with a highly active molecule like aminocyclopyrachlor. In species where de-esterification is rapid, little difference or advantage would be observed between DPX-MAT28 and DPX-KJM44 . In species with relatively slow de-esterification rates, more active ingredient may ultimately be translocated to important organs as
perturbations in the source-sink relationship may be slower to manifest when the herbicide is
applied as DPX-KJM44. Proportionally greater amounts of absorbed DPX-KJM44 than DPX-
MAT28 moved to the roots of rush skeletonweed at 72 HAT. Although initially root-directed,
proportional accumulation of DPX-MAT28 in the roots declined over time.

5 Conclusions

Absorption and translocation of aminocyclopyrachlor is dependent upon active ingredient
form and sensitivity of the target plant species. Highly susceptible species like prickly lettuce
absorb and translocate less material than relatively less sensitive species like rush skeletonweed.
Absorption is enhanced using DPX-KJM44 in all species studied. The least difference in
absorption was observed in rush skeletonweed. Translocation is similar to other auxin phloem
mobile herbicides, accumulating in meristematic sink tissues. De-esterification of DPX-KJM44
in rush skeletonweed appears be a limiting factor in early translocation. Reduced de-
esterification rates may explain the delay in translocation of the resulting acid in yellow
starthistle and rush skeletonweed. Determining sensitivity and de-esterification rates for a target
species will be important in assessing the utility of using the DPX-KJM44. In most instances,
DPX-MAT28 may be sufficient for effective weed control.

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Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to
synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic
Figure 1. Chemical structures of (1) aminocyclopyrachlor acid and (2) aminocyclopyrachlor methyl ester.
Figure 2. Absorption of foliar applied (A) $^{14}$C-DPX-KJM44 and (B) $^{14}$C-DPX-MAT28 into rush skeletonweed, yellow starthistle, and prickly lettuce, expressed as % of applied. Error bars represent the standard error of the mean where n=8.
Figure 3. Total translocation of foliar applied (A) $^{14}$C-DPX-KJM44 and (B) $^{14}$C-DPX-MAT28 out of the treated leaf of rush skeletonweed, yellow starthistle, and prickly lettuce, expressed as % of applied. Error bars represent the standard error of the mean where n=8.
Figure 4. Translocation of foliar applied (A) $^{14}$C-DPX-KJM44 and (B) $^{14}$C-DPX-MAT28 to the above ground portion of each species, expressed as % of applied. Error bars represent the standard error of the mean where n=8.
Figure 5. Translocation of foliar applied (A) $^{14}$C-DPX-KJM44 and (B) $^{14}$C-DPX-MAT28 to the below ground portion of each species, expressed as % of applied. Error bars represent the standard error of the mean where $n=8$. 

(A) Rush skeletonweed, $y=4.4\exp(-\exp((-x-29.4)/25.8))$ 
R$^2=0.99$

Yellow starthistle, $y=2.4\exp(-\exp((-x-12.3)/9.2))$ 
R$^2=0.99$

Prickly lettuce, $y=0.2\exp(-\exp((-x-4.26)/5.26))$ 
R$^2=0.95$

(B) Rush skeletonweed, $y=2.3\exp(-\exp((-x-5.4)/4.43))$ 
R$^2=0.64$

Yellow starthistle, $y=1.25\exp(-\exp((-x-4.9)/2.9))$ 
R$^2=0.91$

Prickly lettuce, $y=0.003x+0.036$ 
R=0.85
Figure 6. TLC plates of extracted leaf tissue from $^{14}$C treated leaf portions of six plants at 2 and 72 HAT harvest intervals. Color indicates intensity of radioactive signal on a log scale.
Figure 7. De-esterification rate of DPX-KJM44 in rush skeletonweed. Percentage of radioactivity from TLC separations of $^{14}$C-DPX-KJM44 and $^{14}$C-DPX-MAT28 from treated leaves of rush skeletonweed (n=6) at different harvest intervals.
Figure 8. Autoradiographs of prickly lettuce (A), rush skeletonweed (B), and yellow starthistle (C), 24 HAT with foliar applied aminocyclopyrachlor methyl ester.