ENVIRONMENTAL AND CHEMICAL CONTROL OF CONE FORMATION IN PLANTATION-GROWN *ABIES FRASERI*

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ABSTRACT

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Fraser fir (*Abies fraseri*) is economically important as a premium Christmas tree in the United States. Christmas tree producers in the Midwest incur substantial monetary losses because of early and heavy cone production. Ironically, cone production is often sporadic in natural stands and consistent cone production is difficult to achieve in conifer seed orchards. The ability to influence cone formation in *Abies fraseri* is important both for Christmas tree growers who want to reduce costs associated with undesired cone production and for seed orchard managers who require consistent seed production.

Cultural practices or chemical treatments may influence the formation of reproductive buds, but only when applied concurrent with cone bud initiation and differentiation. We surveyed shoot phenology at nine Christmas tree plantations in Michigan and used the data obtained to develop a phenology model that predicts the timing of cone bud differentiation in growing degree days. We used that model to time treatments and interpret results in subsequent experiments.

Heat and drought are important environmental regulators of reproduction in conifers. Therefore, we investigated the effects of irrigation and mulch on cone production in two Fraser fir plantations in Michigan. We also tested the effects of overhead misting for evaporative cooling and polyethylene tenting for solar heating of tree crowns. Irrigation did not affect cone production even under drought conditions, compared to non-irrigated control plots. Mulch during a hot summer reduced cone production by 51%, but mulch had no effect during a cooler summer. Misting decreased average daily maximum temperatures of lateral shoots by 5.0°C, and tenting increased maximum temperatures by 3.8°C, but neither affected cone production.

Gibberellins (GAs) regulate reproductive development in conifers and are used to enhance cone production in conifer seed orchards. We identified several plant growth regulators (PGRs) that inhibit GA biosynthesis and evaluated their effects on cone production and vegetative growth at four locations over a three-year period. Paclobutrazol was the most effective PGR for cone control. When applied by soil injection, it reduced average cone density (cones/tree) by 39% two years after application, compared to control.

Cone production is under complex regulation, and environmental factors interact with internal controls through multiple pathways. Therefore, it is difficult to influence cone formation by modifying the environment to alter tree stress. Cultural practices that reduce heat stress to the roots may reduce cone formation during particularly hot summers, and paclobutrazol or other GA inhibitors may limit unwanted cone formation. Future research should examine the interaction of environmental and chemical controls, as they may have greater influence on cone production when used in combination.

Dedicated to my father.
I miss you, Dad.

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TABLE OF CONTENTS

LIST OF TABLES xi

LIST OF FIGURES xii

CHAPTER ONE
GENERAL INTRODUCTION 1

CHAPTER TWO
REGULATION OF CONE INDUCTION IN *ABIES* AND CLOSELY RELATED CONIFERS (LITERATURE REVIEW) 7
- ABSTRACT 8
- 2.1. INTRODUCTION 8
- 2.2. BIOLOGY OF REPRODUCTIVE DEVELOPMENT IN CONIFERS 9
  - 2.2.1. Stages of strobilus development 9
  - 2.2.2. Timing of strobilus initiation and differentiation 10
  - 2.2.3. Phases of reproductive development 10
  - 2.2.4. Masting 11
  - 2.2.5. Markers of developmental phase change 12
    - 2.2.5.1. Reproductive structures 13
    - 2.2.5.2. Morphological traits 14
    - 2.2.5.3. Vegetative regeneration potential 15
    - 2.2.5.4. Vigor 16
    - 2.2.5.5. Biochemical markers 17
- 2.3. ENDOGENOUS CONTROL OF REPRODUCTION 19
  - 2.3.1. Genetic Control of Reproduction 19
    - 2.3.1.1. Fecundity 20
    - 2.3.1.2. Reproductive onset 20
    - 2.3.1.3. Possible mechanisms 21
      - 2.3.1.3.1. *CO*/*FT* regulatory module 21
      - 2.3.1.3.2. MADS box genes and other transcription factors 22
      - 2.3.1.3.3. miR156/*SPL* module 23
      - 2.3.1.3.4. Sugars 24
  - 2.3.2. Epigenetic regulation 25
  - 2.3.3. Meristem maturation 26
  - 2.3.4. Hormonal regulation of reproductive development 27
    - 2.3.4.1. Gibberellins 28
    - 2.3.4.2. Cytokinins (CKs) 30
    - 2.3.4.3. Auxins 31
    - 2.3.4.4. Abscisic acid 32
    - 2.3.4.5. Ethylene 33
- 2.4. EXOGENOUS REGULATION 33
  - 2.4.1. Tree size 33
  - 2.4.2. Environmental factors 35

        2.4.2.1. Temperature ... 35
        2.4.2.2. Moisture ... 35
        2.4.2.3. Light ... 36
        2.4.2.4. Photoperiod ... 37
        2.4.2.5. Nutrition ... 37
  2.5. MANAGING CONE INDUCTION ... 37
    2.5.1. PGRs ... 38
    2.5.2. Cultural treatments ... 40
        2.5.2.1. Fertilization ... 40
        2.5.2.2. Girdling ... 41
        2.5.2.3. Root pruning and transplanting ... 43
        2.5.2.4. Heat ... 43
        2.5.2.5. Drought ... 44
        2.5.2.6. Possible mechanisms ... 45
    2.5.3. Controlling ratio of male to female cones ... 46
    2.5.4. Seed orchard panmixia ... 47
  APPENDIX ... 49

CHAPTER THREE

PHENOLOGY MODEL IDENTIFIES TIMING OF CONE INITIATION IN FRASER FIR (*ABIES FRASERI*) ... 51
  ABSTRACT ... 52
  3.1. INTRODUCTION ... 52
  3.2. MATERIALS AND METHODS ... 54
    3.2.1. Phase I: Development of phenology model ... 55
    3.2.2. Phase II: Mapping of lateral shoot phenology to model ... 57
  3.3. RESULTS ... 57
    3.3.1. Phase I: Development of phenology model ... 57
    3.3.2. Phase II: Mapping of lateral shoot phenology to model ... 58
  3.4. DISCUSSION ... 59
  APPENDIX ... 61

CHAPTER FOUR

USING IRRIGATION AND MULCH TO CONTROL CONE PRODUCTION IN FRASER FIR (*ABIES FRASERI*) ... 67
  ABSTRACT ... 68
  4.1. INTRODUCTION ... 68
  4.2. MATERIALS AND METHODS ... 70
    4.2.1. Site descriptions ... 70
    4.2.2. Reproductive phenology ... 71
    4.2.3. Irrigation and mulch treatments ... 71
    4.2.4. Environmental data collection ... 72
    4.2.5. Coning and growth data collection ... 73
    4.2.6. Carbon isotope analysis ... 73
    4.2.7. Experimental design and statistical analysis ... 74
  4.3. RESULTS ... 74
    4.3.1. Manton ... 74

4.3.2. Gobles 75
4.4. DISCUSSION 77
APPENDIX 80

CHAPTER FIVE
DOES SHOOT TEMPERATURE REGULATE CONE FORMATION IN FRASER FIR (*ABIES FRASERI*)? 88

ABSTRACT 89
5.1. INTRODUCTION 89
5.2. MATERIALS AND METHODS 91
    5.2.1. Mist-cooling treatment 92
    5.2.2. Tent-heating treatment 93
    5.2.3. Data collection 93
    5.2.4. Data analysis 95
5.3. RESULTS 95
    5.3.1. Cone production 95
    5.3.2. Shoot temperature and soil moisture 96
    5.3.3. Vegetative growth 96
    5.3.4. Foliar and soil analyses 97
    5.3.5. Tree injury 97
5.4. DISCUSSION 98
    5.4.1. Shoot temperature and soil moisture 98
    5.4.2. Growth 98
    5.4.3. Tree Stress 99
    5.4.4. Cone production 99
5.4.5. TREE INJURY 100
APPENDIX 104

CHAPTER SIX
GIBBERELLIC ACID INHIBITORS CONTROL HEIGHT GROWTH AND CONE PRODUCTION IN *ABIES FRASERI* 112

ABSTRACT 113
6.1. INTRODUCTION 113
6.2. MATERIALS AND METHODS 116
    6.2.1. Description of field trials 116
    6.2.2. Treatments 116
    6.2.3. Treatment application 117
    6.2.4. Data collection 117
    6.2.5. Tree sampling and stable isotope analysis 117
    6.2.6. Statistical analysis 118
        6.2.6.1. Effects on cone production 118
        6.2.6.2. Effects on growth and carbon isotope discrimination 119
6.3. RESULTS 119
    6.3.1. Cone production 119
    6.3.2. Growth 120
    6.3.3. Carbon isotope discrimination and nutrient analysis 121
6.4. DISCUSSION 121

APPENDIX 126

CHAPTER SEVEN
OVERALL CONCLUSION 132

LITERATURE CITED 136

LIST OF TABLES

Table 3.1. Site characteristics of study locations in Michigan, 2011. 62

Table 3.2. Fitted parameters and measures of fit and predictive power for phenology models of terminal leader growth and lateral growth in Fraser fir in Michigan Christmas tree plantations. 63

Table 4.1. Site descriptions of study locations in Michigan, 2013. 81

Table 4.2. Mean cone density and growth of Fraser fir trees in response to irrigation. Manton, Michigan. 82

Table 4.3. Mean cone density and growth of Fraser fir trees in response to mulch. Gobles, Michigan. 82

Table 5.1. Treatment effects on cone production in *A. Fraseri*, Benton Harbor, MI, 2014 103

Table 5.2. *A. Fraseri* growth (mean ± SE) by treatment, Benton Harbor, MI, 2014. 103

Table 6.1. Site characteristics of study locations in Michigan, 2013. 127

Table 6.2. Distribution of trees (range [mean]) in each size class and dates of PGR application at study sites in Michigan, 2013. 128

Table 6.3. Mean shoot growth, bud density, and carbon isotope discrimination of Fraser fir trees by size class and treatment across four locations in Michigan, 2013 – 2015. 129

Supplemental Table S6.1. Mean cone density (cones/tree) on Fraser fir trees by size class and treatment at four locations in Michigan. 130

LIST OF FIGURES

Figure 2.1. Times and methods of cone initiation. 50

Figure 3.1. Locations of Christmas tree farms that cooperated in the 2011 – 2013 phenology study in Michigan... 64

Figure 3.2. Fitted and validated (a) 2011 phenology model of leader growth and (b) 2013 phenology model of lateral shoot growth using a beta sigmoid function (BSF)................... 65

Figure 3.3. Mapping of Fraser fir lateral shoot phenology to 2011 phenology model of leader growth... 66

Figure 4.1. Soil moisture under irrigated and non-irrigated Fraser fir trees, measured 30 cm below the soil surface. Manton, MI. 83

Figure 4.2. Average daily soil temperatures under irrigated and non-irrigated Fraser fir trees 84

Figure 4.3. Weekly rainfall and reference evapotranspiration (RET), Gobles, MI............. 85

Figure 4.4. Average maximum daily soil temperatures under mulched and non-mulched Fraser fir trees.. 86

Figure 4.5. Soil moisture under mulched and non-mulched Fraser fir trees, measured 30 cm below the soil surface. Gobles, MI.... 87

Figure 5.1. Micro-sprayer delivering mist for evaporative cooling of upper tree canopy. 104

Figure 5.2. Polyethylene tent used for solar heating of upper tree canopy. 105

Figure 5.3. Average daily temperatures of shoots of Fraser fir trees that were tented, misted, or control. 106

Figure 5.4. Daily maximum and minimum temperatures of shoots of Fraser fir trees that were tented, misted, or control. Minimum temperatures overlap for all treatments........ 108

Figure 5.5. Temperatures of shoots of Fraser fir trees that were tented, misted, or control on (a) warm, sunny (July 5); and (b) cool, cloudy (July 28) days in 2014. 109

Figure 5.6. Soil moisture under tented, misted, or control Fraser fir trees, 2014............... 110

Figure 5.7. Fraser fir tree damage due to intolerance to salts present in well water used for the misting treatment.... 111

Figure 6.1. Mean cone density (cones/tree) on small, medium, and large Fraser fir trees in (a) 2014 and (b) 2015 by treatment across four locations in Michigan.............................. 131

CHAPTER ONE

GENERAL INTRODUCTION

Endemic to the southern Appalachian Mountains, Fraser fir (*Abies fraseri*) trees in natural stands typically begin producing cones (strobili) when they are 15 to 30 years old (Johnson 1980; Witter & Ragenovich 1986). When grown for Christmas trees in plantations in the Midwest, Fraser fir trees produce cones much earlier, often just 3 years after planting (Cregg et al. 2003). Cones form in the upper third of the crown, displacing the lateral branches that generate the dense form desired by consumers in the United States. The aesthetic appeal of trees is further reduced when the mature cones disintegrate in early fall, leaving behind unsightly stalks. These concerns have prompted growers to remove the cones by hand early in the season. The average cost for cone removal at the usual planting density of 1200 trees/acre is $528/acre over the course of the rotation (survey of growers, written communication, 2015). One large grower in northern Michigan expended 9000 man-hours on cone removal in 2010 (C. Maciborski, Dutchman Tree Farms, written communication, January 7, 2011). A grower in west-central Michigan asked workers to count the cones as they removed them, tallying over 32,000 cones in 2011, with a maximum of 1100 cones from a single tree. Rather than picking cones, this grower chose to dispose of over 1000 trees which were so heavily laden with cones as to be deemed unsalable (T. Trechter, Mathisen Tree Farms, verbal communication, October 20, 2011). There is also a physiological cost to early cone production. Expanding cones are strong sinks for carbohydrates and divert large amounts of energy from vegetative growth, resulting in shorter shoots and decreased needle length (Powell 1974; Powell 1977). In 2011, we observed an 18% reduction in shoot growth when cones were left on Fraser fir trees (Crain et al. 2012).

Both genetics and environment are likely responsible for the early and heavy cone production observed on Fraser fir trees in Midwestern plantations. Although it can be grafted, Fraser fir is difficult to propagate vegetatively, and is generally grown from seed collected in the wild. Fraser fir is highly threatened in its native range; by the 1980s, 67% of mature trees had been killed by an exotic pest, the Balsam woolly adelgid (*Adelges piceae*) introduced from Europe in the early 1900s (Pauley & Clebsch 1990; Potter et al. 2005). Younger fir trees are replacing the older trees that have died. The high demand for seed may create an unintended selection pressure for earlier maturation, since much of the available seed may be collected from trees that have reached maturity faster than the surrounding trees. Anecdotal support for this idea comes from North Carolina plantations, where growers report an increase in precocious cone production over the past decade. Managed seed orchards are also becoming more common, and may favor trees that flower earlier, since the goal of breeders is to get seeds as quickly as possible from their orchards.

Environmental conditions also regulate reproduction in conifers (Owens & Blake 1985; Day & Greenwood 2011), which progress through three main developmental phases. In the juvenile phase, only vegetative growth is possible and the tree cannot form reproductive structures. At reproductive onset, a conifer may form strobili, but only in the presence of strong environmental cues. Once reproductive competence is attained, strobili are produced annually, regardless of environmental conditions (Williams 2009), although the latter play a role in regulation of mast-seeding events, during which cone production is particularly heavy and coordinated across large spatial areas (Janzen 1976; Kelly 1994). Most Fraser fir trees producing cones in Christmas tree plantations have reached reproductive onset, but not reproductive competence. This suggests that strong external triggers are responsible for the early cone development, and begs a look at the differences between environmental conditions in natural stands and plantations.

Environmental signaling likely plays an important role in Fraser fir cone production, based on climatic differences between Michigan and the southern Appalachians, and the wide interannual variation in cone production. Endemic to a small region of cool, moist-temperate forest in the southeastern Appalachians (southwest Virginia, western North Carolina, and eastern Tennessee), Fraser fir is found only at elevations above 1200 m. The native soils are shallow and rocky, highly acidic (pH 3.8) and well-drained. Annual precipitation is high (1900 – 2540 mm). During the summer, average daily temperatures are below 16°C and trees are typically engulfed by fog for 30 – 35% of all daylight hours (Beck 1990; Reinhardt & Smith 2008). In Michigan plantations, Fraser fir is grown on sandy soils with pH ranging from 4.6 to 7.7 (Rothstein & Lisuzzo 2006). Annual precipitation is 800 – 1309 mm and mean summer temperatures are 16 – 21°C (NOAA 2010).

With such profound environmental differences between Michigan plantations and the native range of the Fraser fir, it seems unlikely that precocious coning can be fully explained by genotypic changes in the populations from which the seed is sourced. This is consistent with what is known about conifer reproduction in general, much of which comes from research to promote cone production in seed orchards: Tree age, size, hormonal interactions, water and nutrient availability, and temperature are key drivers of cone development (Owens & Blake 1985; Owens 1995). Indeed, a complex interaction of environmental and endogenous signaling governs reproductive development in conifers, resulting in significant fluctuations in cone production from year to year (McDonald 1992; Bonnet-Masimbert & Webber 1995). In addition, we have observed dramatic variation in coning within individual fields of singular seed source and planting date (Crain et al. 2012). This suggests that highly localized environmental signaling regulates cone development.

The details of cone development have been reported for other Abies species, including the closely related *A. lasiocarpa* (Owens & Molder 1977; Owens & Singh 1982; Owens 1984; Owens & Morris 1998). Lateral buds form during the summer of the first year, and differentiate into either vegetative or reproductive buds in late summer, toward the end of lateral shoot elongation. Reproductive buds go dormant until the following year, when the cones emerge in early spring. Gibberellins (GAs) are active in hormonal signaling that triggers reproductive development in conifers (Pharis & Kuo 1977), and are often applied to enhance cone production in conifer seed orchards, where consistent cone production is difficult to attain (Puritch 1979; Owens 1995). High temperatures and drought are correlated with heavy cone production in conifers (Owens & Blake 1985; Solberg 2004), and are used in seed orchards to increase cone production, generally in combination with GA (Ross & Pharis 1985; Smith & Greenwood 1997). However, cultural practices or GA must be in place throughout the period of reproductive bud initiation and differentiation in order to be effective (Owens & Blake 1985).

This dissertation is structured as a collection of papers that have been or will be submitted for publication in Forest Science (chapters 2 and 3) or Scandinavian Journal of Forest Research (chapters 4 – 6). Chapter 2, the literature review, provides information on what is currently known about regulation of reproductive development in Fraser fir and other north temperate conifers, and forms the basis for the dissertation research. In chapter 3, we introduce a phenology model of shoot growth in Fraser fir that predicts the timing of reproductive bud initiation and differentiation based on growing degree days. This model was used to time treatments and interpret results described in subsequent chapters. Chapters 4 and 5 examine the effects of environmental stress on cone production. Preliminary research indicated a negative correlation between soil moisture content and cone production, as well as associations between temperature and cone production. These findings are consistent with the correlations between drought and heat stress and conifer reproduction noted in the literature (e.g., Owens & Blake 1985). Therefore, it seems likely that early and heavy cone production may be triggered by the warmer and drier climate in Michigan and the Upper Midwest, compared to the southern Appalachians. Chapter 4 examines the use of irrigation and mulch to control cone production in Fraser fir by mitigating the promotive effects of drought and heat stress on cone formation. In chapter 5, we describe the effects of evaporative cooling and solar heating of the tree canopy on cone production. Cone production is also under hormonal control; chapter 6 presents a study evaluating the effects of plant growth regulators (PGRs)—particularly GA biosynthesis inhibitors—on cone production. Finally, we close with some general conclusions and recommendations (Chapter 8).

CHAPTER TWO

REGULATION OF CONE INDUCTION IN *ABIES* AND CLOSELY RELATED CONIFERS

(LITERATURE REVIEW)

ABSTRACT

REGULATION OF CONE INDUCTION IN *ABIES* AND CLOSELY RELATED CONIFERS (LITERATURE REVIEW)

Forest tree improvement programs provide the basis for most of our knowledge of cone induction in conifers. Since World War II, forest geneticists and tree breeders have largely selected for traits that improve productivity, and improved seed is now commonly produced in seed orchards. However, seed production in many conifers varies widely from year to year, regulated in part by environmental conditions. Therefore, much of the research in conifer reproduction has focused on enhancement of cone development during summers when weather conditions are unfavorable to reproductive bud initiation. In this review, we synthesize current knowledge about the manipulation of cone production in north temperate conifers. We provide an overview of conifer reproductive biology, describe the progression from juvenility to reproductive maturity, and discuss both endogenous (e.g., genetic, epigenetic, hormonal) and exogenous (e.g., temperature, moisture, tree size) regulation of reproductive development. Finally, we summarize the most common approaches to cone enhancement in seed orchards and suggest possible mechanisms that might govern reproductive development, such as the GA-DELLA and miR156/*SPL* modules.

2.1. INTRODUCTION

Much of what we know about strobilus induction in conifers stems from tree improvement programs that service the timber and pulpwood industries. Since World War II, forest geneticists and tree breeders have systematically selected for traits that improve wood quality and productivity (i.e., growth rate). Improved seed is commonly produced in seed orchards (Miller & DeBell 2013). However, many conifers are mast-seeding species, producing copious amounts of seed across broad geographic areas under favorable conditions, only to be followed by several years of low seed production (Kelly 1994). Considerable research has thus centered on enhancement of cone development under unfavorable conditions. This review attempts to synthesize what we have learned about manipulation of cone production in *Abies* and related north temperate conifers.

2.2. BIOLOGY OF REPRODUCTIVE DEVELOPMENT IN CONIFERS

2.2.1. Stages of strobilus development

Strobilus development in conifers is a complex, multi-stage process. Three distinct stages—induction, initiation, and differentiation—are of particular importance in understanding cone development. During strobilus induction, the meristem in the lateral bud is reprogrammed to transition from vegetative to reproductive growth. This stage is identifiable by the upregulation of certain proteins and nucleic acids required for further development. The second stage, strobilus initiation, is characterized by the first detectable morphological changes (shape or size) within the meristem, which reorganizes to prepare for the production of reproductive structures. The third stage in early strobilus development is differentiation, which is marked by the organogenesis of reproductive structures and represents an irreversible change that persists even if the tree is released from the conditions that originally promoted strobilus induction (Sedgley & Griffin 1989).

We will follow the lead of Owens and Blake (1985) in using initiation to refer to the first two stages (induction and initiation) combined. This is to avoid confusion, because induction is commonly used in the literature to refer to promotion of cone development by chemical or cultural means, such as in a seed orchard (e.g., Owens & Blake 1985). Also, the precise timing of induction has not been determined for most species, since it precedes any morphological change and is therefore more difficult to detect than subsequent stages.

2.2.2. Timing of strobilus initiation and differentiation

The timing of strobilus initiation and differentiation is governed by thermal time (growing degree days) and varies by genus and species. In *Abies,* axillary bud primordia develop on newly expanding shoots about one week prior to vegetative bud-break (Powell 1974; Owens & Blake 1985). Bud-scale initiation commences, and continues for several weeks, coinciding with shoot elongation. Strobilus initiation occurs in early summer, when lateral shoot elongation begins to slow, towards the end of bud-scale initiation. Biochemical and anatomical changes occur over the next several weeks, with differentiation complete near the end of lateral shoot elongation. The timing of strobilus initiation and differentiation is essentially the same for *Pseudotsuga* and *Picea*, with differentiation complete by the end of bud-scale initiation and lateral shoot elongation (Owens & Blake 1985). Strobilus development for many conifers is summarized in Figure 2.1.

2.2.3. Phases of reproductive development

Conifers change in their sensitivity to environmental signaling as they progress through the three phases (juvenile, adult vegetative, and adult reproductive) of post-embryonic development (Poethig 1990; Greenwood 1995). In the juvenile phase, only vegetative growth is possible and the conifer cannot form reproductive structures, even under strongly inductive conditions that would otherwise result in reproductive development. At *reproductive onset*, a conifer enters the adult reproductive phase and may form strobili, but only in the presence of strong external cues, such as drought stress or exogenous application of GA. *Reproductive* *competence*—the point after which strobili are produced regularly, regardless of environmental conditions—marks entry into the *adult reproductive phase* (Bond 2000; Williams 2009).

Advancing through these phases is a long, complex process in conifers that enables them to respond to changes in environment, size, and complexity as their life cycle progresses (Day & Greenwood 2011). It is not governed by a single, regulatory event, but is facilitated by multiple, overlapping processes that affect both vegetative and reproductive development (Hackett & Murray 1993). Gene expression, operating at the level of the meristem, interacts with internal and external factors to regulate morphological and physiological changes that offer strategic advantages specific to the current life stage (Poethig 1990; Day & Greenwood 2011).

Although ontogeny is generally thought of in terms of whole tree development, individual meristems within a tree may exhibit different degrees of maturation (Wendling et al. 2014a). Meristem maturation involves complex changes that alter the meristem's response to internal and external signals, engendering reproductive competence and morphological changes, while reducing regenerative potential and vigor (Trewavas 1983; Hackett 1987; Hutchison et al. 1990; Greenwood 1995; Poethig 2003).

2.2.4. Masting

Many conifers are mast-seeding species, meaning that seed production is highly variable across years, but synchronized across a population (Janzen 1976; Kelly 1994). Depending on the species, the synchrony may be detectable across sites for 100s or even 1000s of km (Koenig & Knops 1998), although plasticity in response to local site conditions is evident (Crone et al. 2011; Roland et al. 2014). Mast seeding comes at the expense of vegetative growth, and is thought to be an evolutionary strategy that involves mass-scale reproductive output to increase fitness by, for example, satiating seed predators (Janzen 1976) and improving wind pollination

(Kelly 1994; Koenig & Knops 1998). Weather and the availability of resources, such as nutrients, water, and light, likely serve as environmental signals that synchronize mast seeding. Long-term accumulation of resources, such as nutrients and photosynthates, may create an additional, internal trigger (Koenig & Knops 2000). Sensitivity to these factors varies by species. In general, trees accumulate resources during periods conducive to vegetative growth, when rainfall and temperatures are in the optimal range. The availability of sufficient reserves within the tree then creates a permissive state for reproductive development. Subsequent hot, dry conditions during bud development encourage strobilus initiation and differentiation. A mast year with heavy cone production follows one to two years later, depending on the length of the reproductive cycle (Owens & Blake 1985; Roland et al. 2014). Growing and developing strobili are strong sinks for photosynthates and other resources, such as nitrogen and phosphorus. Reserves must be replenished in order to repeat the masting cycle (Powell 1977; Sala et al. 2012). In biennially bearing species, such as balsam fir (*Abies balsamea* [L.] Miller), cone production generally occurs every other year, indicating that the temporal pattern of cone production is under endogenous control. In contrast, the number of strobili produced in a mast year is highly regulated by weather and resource availability. The biennial pattern is most simply explained by source-sink relations: Initiation of new strobili is likely limited by competition for local resources among existing, growing and developing cones, which are a strong sink for photosynthates (Powell 1977).

2.2.5. Markers of developmental phase change

Much of the information on developmental phase change in plants is provided by studies in model systems, such as *Arabidopsis*, in which flowering may occur just weeks following germination. In long-lived conifers, phase change transitions may occur over months or years, and it may be decades before full reproductive competence is attained. The ability to gauge maturation state is critical in clonal forestry and tree improvement programs, where mature material must often be rejuvenated in order to propagate trees with desirable traits. Phase change indicators in forest trees have been comprehensively reviewed by Wendling et al. (2014a), and include decreased regeneration potential, changes in foliar and stem properties, changes in vegetative vigor and habit, and various hormonal, physiological and biochemical changes. These gauges give an approximate idea of the developmental state, particularly where strong correlations have been noted between the marker and developmental phase in a particular species. However, such indicators are often imprecise and may confound vegetative and reproductive phase change, which often overlap, but appear to be genetically and physiologically distinct (Wiltshire et al. 1994; Hasan & Reid 1995; Abedon et al. 1996; Wiltshire et al. 1998; Jordan 1999). Plastic responses to environmental signals may also mimic ontogenetic morphological or physiological changes. Identification of a highly conserved biochemical marker would greatly improve the rate and precision of scientific discovery in the area of phase change in woody plants (Wendling et al. 2014a).

2.2.5.1. Reproductive structures

At present, the most reliable indicator of phase change is the formation of reproductive structures. In conifers, the first production of strobili marks reproductive onset, and a regular, temporal pattern of cone production indicates reproductive competence. In some conifers, the ratio of male to female strobili is a useful indicator of maturation (Greenwood & Hutchison 1993). In each case, the presence of strobili indicates that the phase transition has already occurred physiologically, resulting after some time in morphologically identifiable structures.

Likewise, the lack of strobili does not necessarily indicate a lack of reproductive capacity, since multiple pathways regulate cone formation.

Strobilus formation is also affected by crown position: Megastrobili typically form in the upper region of the crown, but rarely in the lower crown. Shoots lower in the tree exhibit additional juvenile traits, compared to shoots higher in the tree, such as the ability to readily form adventitious roots (Grace 1939; Hackett 1987).

2.2.5.2. Morphological traits

Various morphological and physiological changes may occur as a tree progresses from juvenile to adult vegetative phase (Poethig 1990). Prior to onset of reproductive structures, these traits offer the best gauge of maturation state (Wendling et al. 2014a). Needle and stem morphology, such as increasing needle width with age, are useful indicators of phase change in temperate conifers. However, in reciprocal grafting studies, red spruce (*Picea rubens* Sarg.) needle characteristics were influenced by both scion and rootstock age, suggesting interactions between multiple control pathways (Day & Greenwood 2011). This limits their usefulness in rejuvenation efforts, where needle morphology may not accurately predict other age-related traits, such as rooting potential.

Also, it is important to differentiate ontogenetic traits—which develop in a predictable pattern independent of environmental conditions (Poethig 2013)—from plastic traits, such as morphological changes during organogenesis in response to environmental cues. For example, it has long been known that leaf morphology of heteroblastic plants changes as the plants mature (Goebel, 1900). For these plants, leaf morphology may be used as an ontogenetic marker. However, leaves on plants grown in shade may exhibit juvenile morphological traits. This has been interpreted as evidence that shade slows whole plant development, increasing the duration of juvenility (Goebel, 1908; Njoku, 1956). Jones (1995) was the first to demonstrate that developmental plasticity at the level of the individual leaf could account for the morphological changes attributed to juvenility. The shape of the leaf was thus a function of light interception during development, not delayed maturation, even though the leaf morphologically resembled a juvenile leaf. It is not easy to separate out which factors are actually influencing the rate of maturation, and not simply triggering a local plastic response to environmental conditions.

2.2.5.3. Vegetative regeneration potential

Woody plants—especially conifers—generally exhibit declining vegetative regeneration potential as the tree matures (Greenwood 1995). In many species, roots or shoots may be induced adventitiously from the vascular parenchyma or cambial cells in juvenile cuttings (Díaz-Sala et al. 1996; de Klerk et al. 1999; Ballester 1999). This competence is lost with age, and mature cuttings may not readily form roots even in response to exogenous application of auxins (Geneve & Kester 1991; Ballester 1999; Díaz-Sala 2014).

Rejuvenation techniques—such as tissue culture, serial grafting, and hedging—are used in tree improvement programs, where propagation from mature cuttings is often necessary (Hackett 1985; Greenwood 1987; Greenwood 1995). Tissue culture involves culturing mature explants *in vitro*, generally in the presence of a cytokinin, such as benzyladenine (BA). Newly formed plantlets retain mature traits, and serial subculture (repeatedly culturing cuttings of new plantlets) is required before juvenile traits are restored. Similarly, serial grafting results in the gradual restoration of juvenile traits by recurrent grafting of scions onto juvenile rootstock. Juvenile traits may be maintained in some species, such as Norway spruce (*Picea abies* L. Karst.) and Monterey pine (*Pinus radiata* D. Don), by annual hedging, which stimulates production of shoots with high rooting capacity (Greenwood 1987).

It is unclear to what degree rejuvenation attempts result in true rejuvenation (reduced maturation), rather than just reinvigoration (increased vigor). Frequently, regenerative potential is temporarily restored, while other mature traits remain (Greenwood 1995; Wendling et al. 2014b). Thus regenerative potential should not be used as the sole marker for maturation state, without consideration of additional traits (Hackett 1985; Greenwood 1987). For a thorough review of juvenility maintenance and methods of rejuvenation, see Wendling et al. (2014b).

2.2.5.4. Vigor

As they age, temperate conifers decrease in shoot vigor (Greenwood 1995), which inversely correlates with increasing reproductive capacity (Day & Greenwood 2011). Thus, vigor is often useful as an indicator of maturation state. However, in grafting studies on red spruce (Greenwood et al. 2010), juvenile scions produced more vegetative growth than did older scions only when grafted to juvenile rootstock, and total vegetative growth was similar for scions of all ages when grafted onto middle-aged or old-growth rootstock. Growth habit was different, however: Middle aged rootstock promoted terminal shoot elongation, and old growth rootstock promoted branch density. This suggests that juvenile meristems have greater growth potential that may be limited by distance to the roots as the tree grows taller, but that growth potential does not continue to decrease as the tree further matures from middle aged to old growth. This is consistent with work on several tree species, including Scots pine (*Pinus sylvestris* L.) (Mencuccini et al. 2005; Vanderklein et al. 2007) and Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) (Bond et al. 2007), which established that decreases in shoot growth in later life stages are not due to decreased vigor in more mature meristems, but are a function of tree size—a factor extrinsic to the meristem. This evidence suggests that vigor may be useful as an indicator of phase change early in the life history of a tree, but growth habit may become more important in later years.

Additionally, the growth rate of more mature grafts matches that of juvenile grafts after the first growing season, suggesting both that the growth advantage of juvenility decreases with tree size, and that mature scions may be reinvigorated (Greenwood et al. 1989; Bond et al. 2007; Mencuccini et al. 2007). In Douglas fir, all scions of all ages from juvenile to old growth exhibit the vegetative growth traits of the rootstock within 2 years of grafting (Bond et al. 2007). This suggests that vegetative vigor—although still useful as a proxy—is regulated quite differently than reproductive development, which is related to changes in the mature meristem, and generally irreversible in grafting studies (Bond et al. 2007; Greenwood et al. 2010; Day & Greenwood 2011).

2.2.5.5. Biochemical markers

Maturation in woody plants is a complex process involving changes in levels of numerous metabolic compounds and gene products, many of which have been evaluated as potential markers of phase change (Haffner et al. 1991). Yet efforts to identify simple and reliable biochemical phase change markers in forest tree species have not been successful (Wendling et al. 2014a). Many potential markers, such as differentially expressed gene products, appear to be species-specific (von Aderkas & Bonga 2000). Others are involved in intricate crosstalk between multiple pathways, requiring complex analysis of multiple parameters that are difficult to interpret (Haffner et al. 1991). A reliable marker would greatly simplify many forest tree improvement tasks, such as monitoring rejuvenation efforts or selecting appropriate material for seed orchards. Ideally, the marker would not only identify the current developmental phase of a forest tree, but would gradually change in gradient according to developmental progression from one phase to the next (von Aderkas & Bonga 2000; Valdés, Fernández, et al. 2003; Valdés, Centeno, et al. 2003).

Hormones are important regulators of phase change in plants (Trewavas 1983; Haffner et al. 1991; Valdés et al. 2002; Davies 2010; Turnbull 2011). Unfortunately, individual hormone levels can fluctuate dramatically due to crosstalk between signaling pathways and simultaneous involvement in multiple processes. Although individual hormones may be unsuitable as markers, certain endogenous hormone ratios may correlate with maturation, but this varies by species. For example, in Monterey pine (Valdés et al. 2002)—but not Stone pine (*Pinus pinea* L.) (Valdés et al. 2004)—the ratio of isopentenyladenine-type to zeatin-type cytokinins decreases as the tree matures, and increases during reinvigoration by grafting (Valdés, Fernández, et al. 2003).

Polyamines (PAs) are small, broadly conserved polycations that function in diverse cellular processes, including growth, development, and stress response (Kusano et al. 2008; Takahashi & Kakehi 2010). Because PAs are upregulated in actively growing and dividing cells, they are differentially expressed in juvenile and mature tissues (Kumar et al. 1997), making them potential indicators of phase change in woody plants (Rey et al. 1994). In peach (*Prunus persica* [L.] Batsch) and Monterey pine, both free putrescine (an important PA) and the ratio of free PAs to conjugates decrease as the tree matures (Fraga et al. 2004). However, PA metabolism varies greatly among species (Fraga et al. 2004; Wendling et al. 2014a) and in response to seasonal growth patterns and stress (Königshofer 1989), which could complicate its use as an ontogenetic gauge.

The most promising candidate for a molecular marker is microRNA156 (miR156). miRNAs are small, non-encoding RNAs that regulate numerous important processes in plants, including vegetative phase change (Wang et al. 2011), flowering time (Aukerman & Sakai 2003; Chen 2004), and biotic (Katiyar-Agarwal & Jin 2010) and abiotic (Sunkar et al. 2007) stress responses. miR156 has emerged as a general regulator of vegetative phase change in herbaceous (Wu & Poethig 2006; Xie et al. 2006; Chuck et al. 2007; Xie et al. 2012; Fu et al. 2012; Salinas et al. 2012; Shikata et al. 2012) and woody plants (Wang et al. 2011). The expression level of miR156 is highest when the plant is young, and decreases as the plant ages (Wu & Poethig 2006; Xie et al. 2006; Chuck et al. 2007; Wu et al. 2009; Jung et al. 2012). It is therefore possible to use miR156 as a molecular marker to distinguish between juvenile and adult plants (Poethig 2013), but it remains unknown how well miR156 levels correlate with subtle changes in morphology and physiology associated with maturation. In Cole's wattle (*Acacia colei* Maslin & L.A.J.Thomson), high levels of miR156 correlated with juvenile leaf morphology, intermediate levels correlated with transition leaf morphology, and low levels correlated with adult leaves (Wang et al. 2011). Thus it may be possible to gauge incremental changes in a tree's maturation by monitoring changes in miR156 expression, long before the development of reproductive structures—or even the competence to form such structures. miR156 is conserved in gymnosperms (Qiu et al. 2009; Huijser & Schmid 2011), but to what degree ontogenetic pathways are conserved is unknown.

2.3. ENDOGENOUS CONTROL OF REPRODUCTION

2.3.1. Genetic Control of Reproduction

Both fecundity and the timing of reproductive onset in plants are mediated through intricately controlled gene expression (Hackett 1985; Greenwood & Hutchison 1993; Greenwood 1995; Cardon et al. 1999; Greenwood et al. 2010; Wendling et al. 2014a). Genotype establishes the general parameters for reproductive development at the whole tree level, and at the level of the individual meristem (Diggle 1993; Day & Greenwood 2011). Various intrinsic and extrinsic factors regulate the expression of genes that mediate phase change, influencing the fate of cells produced by the meristem (Day et al. 2002).

2.3.1.1. Fecundity

Evidence for genetic control of fecundity comes from forestry tree improvement research. In red spruce seed orchards, strobilus production among clones may differ by orders of magnitude, which cannot be accounted for by variation in environmental variables (Greenwood et al. 2010; Day & Greenwood 2011). In loblolly pine (*Pinus taeda* L.), greater than 50% of female and 40% of male reproductive output may be attributable to genetic effects (Schmidtling 1983).

2.3.1.2. Reproductive onset

A number of genetic studies have demonstrated that time to reproductive onset is an inheritable trait in woody plants (Johnsson 1949; Stern 1961; Visser 1976), including conifers (Heimburger & Fowler 1969; Johnson & Critchfield 1978). Evidence for genetic control of maturation in conifers is provided by studies following a common-rootstock approach, in which scions from juvenile or mature trees are grafted onto rootstock of equal age. This approach controls for the age and size of the tree, as well as for environmental conditions. Grafted scions from Monterey pine (Sweet 1973), loblolly pine (Greenwood 1984), eastern larch (*Larix laricina* [Du Roi] C. Koch) (Greenwood et al. 1989; Hutchison et al. 1990), red spruce (Rebbeck et al. 1993), and Douglas fir (Ritchie & Keeley 1994) exhibited foliar morphological and physiological traits consistent with the parental ontogeny, persisting for at least several years.

2.3.1.3. Possible mechanisms

In *Arabidopsis*, flowering time is regulated by photoperiod, temperature and endogenous signaling primarily through five independent, but partially overlapping, genetic pathways: photoperiodic, vernalization, autonomous, gibberellin, and vegetative phase change pathways (Koornneef et al. 1991; Simpson & Dean 2002; Mencuccini et al. 2005; Bäurle & Dean 2006; Lee & Lee 2010; Amasino & Michaels 2010; Srikanth & Schmid 2011). Although independent, these pathways interact to regulate flowering. For example, the vernalization and vegetative phase change pathways do not induce flowering directly, but create the permissive state required for induction of flowering through the photoperiodic pathway (Poethig 2013).

In gymnosperms, little is known about the pathways that govern reproduction, but at least some of the genetic mechanisms found in *Arabidopsis* are conserved in woody plants, including conifers (Castillo et al. 2013; Uddenberg et al. 2013; Wendling et al. 2014a). Some of the more than 50 genes known to regulate flowering time in *Arabidopsis* (see Srikanth & Schmid 2011) are known to exist in conifers (Purugganan 1997; Sundstrom et al. 1999; Carlsbecker et al. 2004; Carlsbecker et al. 2013), suggesting possible mechanisms for genetic control of ontogeny in conifers.

2.3.1.3.1. *CO*/*FT* regulatory module

FLOWERING LOCUS T (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) are floral integrators, meaning they integrate signals that converge from multiple flowering pathways (Amasino & Michaels 2010). A growing body of evidence suggests that the FT protein is a mobile, florigenic signal conserved among all flowering plants (Samach et al. 2000; Putterill et al. 2004; Lifschitz et al. 2006; Yang et al. 2007; Turck et al. 2008). In the *Arabidopsis* photoperiodic flowering pathway, *CONSTANS (CO)* activates *FT*, which upregulates *SOC1*, which in turn activates floral meristem identity genes that initiate flowering (Koornneef et al. 1991; Wigge et al. 2005; Yoo et al. 2005). The *CO/FT* regulatory module has been shown to regulate the timing of both reproductive onset and photoperiodic bud-set in *Populus* spp. (Böhlenius et al. 2006). Recently, *FT* orthologs were found to regulate bud-set in Norway spruce (Gyllenstrand et al. 2007) and Scots pine (Avia et al. 2014), suggesting that the *CO/FT* regulatory module is conserved in conifers. This module may control reproductive development by upregulating florigenic FT protein as the tree matures (Day & Greenwood 2011).

2.3.1.3.2. MADS box genes and other transcription factors

Flowering pathways converge on *SOC1* and other integrators that promote floral transition of the meristem by activating transcription factors, notably *LEAFY* (*LFY*) and the MADS box genes *FRUITFULL* (*FUL*) and *APETALA1* (*AP1*) (Benlloch et al. 2007; Yamaguchi et al. 2009). Homologs of *LFY* have been found in gymnosperms and implicated in reproductive identity of the meristem, but expression patterns differ from those of angiosperms, and their activity and role are less well characterized (Benlloch et al. 2007). In Norway spruce, MADS box genes have been identified that are believed to regulate the duration of juvenility and the reproductive or vegetative identity of a meristem (Carlsbecker et al. 2003; Carlsbecker et al. 2004). Expression of *DEFICIENS AGAMOUS-LIKE1* (*DAL1*) is upregulated as the tree ages, and distributed in a pattern that precisely predicts the changes in morphological and physiological traits that occur during reproductive phase change. Also, juvenility is shortened or non-existent in transgenic *Arabidopsis* expressing *DAL1* (Carlsbecker et al. 2004). A related gene, *DAL10*, appears to regulate reproductive identity in Norway spruce (Carlsbecker et al. 2003). Phylogenetic analysis indicates that *PaDAL19* (in Norway spruce) and *AtSOC1* (in

Arabidopsis) are in orthologous clades, and *PaDAL19* is upregulated in reproductive shoots (Uddenberg et al. 2013), which may suggest similar mechanisms in specification of reproductive identity.

2.3.1.3.3. miR156/*SPL* module

SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (*SPL*) genes encode transcription factors that regulate gene expression to control many aspects of plant development, including floral transition (Cardon et al. 1997; Cardon et al. 1999; Poethig 2013). This age-related floral transition pathway—also known as the vegetative phase change pathway (Poethig 2013)—is regulated by miR156, which inhibits flowering during the juvenile phase (Wang et al. 2009). The primary function of miR156 is to repress *SPL* gene expression (Poethig 2013). miR156 levels are high in juvenile plants and decrease over time, while SPL protein levels are low in juvenile plants and increase over time. Mutations that prevent miR156 from binding to SPL gene transcripts result in precocious flowering (Cardon et al. 1997; Wu & Poethig 2006; Gandikota et al. 2007; Usami et al. 2009; Yamaguchi et al. 2009; Wang et al. 2009). Despite the high functional redundancy within the gene family, loss-of-function mutations of certain *SPL* genes result in delayed flowering and extended juvenility (Schwarz et al. 2008; Wang et al. 2008). Constitutive overexpression of miR156 by the 35S promoter likewise prolongs juvenility in the poplar hybrid *Populus x canadensis* by suppressing expression of two *SPL* genes (Wang et al. 2011).

miR156 maintains juvenility and governs the phase change transition whereby a meristem becomes competent to flower, but miR156 does not induce flowering (Poethig 2013). The decline in miR156 as the plant ages releases *SPL* from miR156-mediated suppression, creating a permissive state for other pathways (vernalization, photoperiodic, and GA) to regulate

SPL transcription and thus control floral initiation (Wang et al. 2009; Jung et al. 2011; Jung et al. 2012; Yu et al. 2012). In *Arabidopsis*, *SPL* functions in parallel with *CO* to upregulate *FT* (Yu et al. 2012), and also directly activates MADS box genes in the meristem to initiate flowering (Wang et al. 2009). miR156 is conserved in gymnosperms (Huijser & Schmid 2011; Zhang et al. 2012; Wang & Wang 2015). Indeed, its recent discovery in the unicellular alga *Chlamydomonas reinhardtii* indicates that its role in gene silencing traces back to primitive eukaryotes (Molnár et al. 2007).

Levels of many miRNAs involved in growth and development change in response to biotic and abiotic stress signals (Sunkar & Zhu 2004; Fujii et al. 2005; Navarro et al. 2008; Khraiwesh et al. 2012; Sunkar et al. 2012; Kruszka et al. 2012), suggesting integration of stress-response and developmental pathways (Sunkar et al. 2012). For example, miR156 is upregulated in response to heat (Lee et al. 2010; Kim et al. 2012; Stief et al. 2014), drought (Sun et al. 2012), nutrient deficiency (Hsieh et al. 2009), and salt stress (Ding et al. 2009; Sun et al. 2012), resulting in a corresponding decrease in SPL levels. These stress-mediated changes in miRNA expression levels may account for reduced growth and development in response to stress (Sunkar et al. 2012).

2.3.1.3.4. Sugars

In *Arabidopsis*, leaf removal results in delayed phase change and increased miR156 expression. This suggests that a repressive signal from the leaves inhibits miR156 (Yang et al. 2011). Nutrients—especially sugars—have long been implicated in phase change due to their effects on morphology and physiology in heteroblastic plants (Goebel 1900; Goebel 1908; Allsopp 1952; A Allsopp 1953; A. Allsopp 1953; Feldman & Cutter 1970; Njoku 1971). Sugars accumulate rapidly in the meristem prior to initiation of flowering in *Arabidopsis*, and could serve as a mobile signal (Eriksson et al. 2006). Vegetative phase change is delayed in chlorophyll-deficient *Arabidopsis* mutants, chlorina1-4 (*ch1-4*; Yang et al. 2013; Yu et al. 2013). This delay results from decreased production of sugars, and is mediated by HEXOKINASE1 (HXK1), a glucose signaling protein that helps maintain juvenility when sugar levels are low, such as under low-light conditions. HXK1 acts by repressing MIR156 transcription in response to glucose signaling (Yang et al. 2013). The gradual accumulation of sugars from seed germination to maturation may serve as an endogenous aging signal that regulates developmental timing by downregulating miR156 as the plant ages (Yu et al. 2013).

2.3.2. Epigenetic regulation

Epigenetic regulation results in phenotypic changes by persistently modifying gene expression without altering the underlying DNA (Yakovlev & Fossdal 2012). Many of these changes are not transmitted to progeny. Examples include changes in foliar morphology and branching habit with increasing age and size (Greenwood et al. 2010). In contrast, epigenetic inheritance is transgenerational, adjusting progeny performance for many years, based on the parental environmental conditions—especially temperature, photoperiod, and irradiance—at the time of seed initiation and development (Day & Bonduriansky 2011; Yakovlev et al. 2011; Yakovlev & Fossdal 2012). These aftereffects vary by trait and among species (Andersson 1994). For example, a colder maternal environment results in earlier bud break and bud set, which are important in cold hardiness (Johnsen et al. 2005). The timing of bud set also regulates reproductive bud initiation and differentiation in conifers. A slight delay may expose the developing bud to warmer conditions, thus encouraging reproductive over vegetative development (Owens & Blake 1985; Day & Greenwood 2011). Plants are particularly sensitive to changes in environmental conditions when juvenile and during sporogenesis and gametogenesis (Bazhina 2014). Seed orchards must be carefully sited, or action must be taken to modify the microclimate around the trees during spore and seed development to prevent undesired epigenetic effects on progeny (Schmidtling 1987; Funda & El-Kassaby 2013).

2.3.3. Meristem maturation

Day et al. (2002) describe intrinsic and extrinsic controls that regulate ontogeny. Intrinsic controls modify gene expression in the meristem through epigenetic changes, such as DNA-methylation, histone modification, and telomere shortening. The signals prompting these changes may initiate in the meristem, or follow transduction pathways from the external environment (Day & Greenwood 2011). Grafting studies on red spruce indicate that reproductive phase change is primarily determined by the maturation state of the meristem (Greenwood et al. 2010), which is largely the result of epigenetic changes to gene expression that are generally permanent and irreversible (Day & Greenwood 2011). Other epigenetically-governed traits, such as branching habit, may be partially reversed by altering external conditions (Greenwood et al. 2010).

It is difficult to separate size effects from age (maturation) effects, but progress has been made in recent years. Changes in shoot elongation are largely a function of tree size. Foliar morphology and branching habit are governed by both size and meristem maturation. Reproductive development is primarily regulated by meristem maturation (Bond et al. 2007; Greenwood et al. 2010). This is important for seed orchard management, because cone-inducing treatments differentially affect meristems based on degree of maturation. Juvenile meristems rarely respond to such treatments (Day & Greenwood 2011).

2.3.4. Hormonal regulation of reproductive development

Plant development is regulated by hormone signaling pathways that integrate endogenous and exogenous cues through complex, overlapping regulatory networks. Hormone-mediated developmental and biotic and abiotic stress response pathways interact to precisely control the final gene expression, generating a robust, yet dynamic system that enables developmental plasticity in response to changing environmental conditions (Achard et al. 2007; Vanstraelen & Benková 2012). miRNAs have emerged as important mediators of this crosstalk between hormone signaling pathways (Curaba et al. 2014). The miR156/*SPL* module integrates environmental signals into the vegetative and reproductive phase change pathways it regulates. miR156 levels are primarily determined by age and decrease over time (Yamaguchi & Abe 2012), but may be influenced by auxin (Marin et al. 2010) and ethylene (Zuo et al. 2012) in some instances, and are upregulated in response to stress (Sunkar et al. 2012). miR156 maintains juvenility by repressing SPLs (Poethig 2013). GA, auxin, ethylene, and abscisic acid (ABA) all regulate the stability of DELLA proteins that target some of the same SPLs (Vanstraelen & Benková 2012). Thus hormone signaling may serve to dynamically integrate environmental information into developmental programming through the miR156/*SPL* module (Wang & Wang 2015).

Plant hormones are important regulators of maturation and reproductive development in temperate conifers (Haffner et al. 1991; Greenwood 1995; Valdés et al. 2002). Mitotic activity, controlled by auxin, CK, and GA, maintains the stability of the developmental state of the meristem (Haffner et al. 1991; Valdés et al. 2002; Vanstraelen & Benková 2012). Auxin, CK, and strigolactones regulate shoot branching, and GAs and brassinosteroids add additional control to tune the levels of auxin and CK in several developmental processes (Vanstraelen &

Benková 2012). Hormonal changes during strobilus initiation and differentiation suggest a role in regulating gender determination (Chałupka 2008; Kong et al. 2012) and strobilus distribution within the crown, although responses vary by genotype (Chałupka 2008).

2.3.4.1. Gibberellins

The role of GAs in reproductive development has been extensively studied in temperate conifers for tree improvement purposes (Owens 1995). In Cupressaceae and Taxodiaceae, GA_3 promotes strobilus formation. In Pinaceae, the less polar GAs, such as GA_4 and GA_7, are promotive. In addition, the ratio of polar to less polar GAs decreases with age, suggesting a role for GA in regulating phase change (Pharis & Kuo 1977). These less polar GAs are important in reproductive organogenesis and accumulate at the meristem from neighboring tissues immediately before strobilus initiation. Indeed, induction of strobili by exogenous application of $GA_{4/7}$ is possible in many species. Similarly, certain GAs—especially less polar GAs—may be upregulated in response to environmental stresses that promote cone production, such as drought and heat stress, whether naturally occurring or culturally induced in seed orchards (Pharis & Kuo 1977).

The mechanism by which GAs regulate strobilus initiation or differentiation (e.g., Smith 1998) is not known, but similarities shared with angiosperms and other plants suggest that molecular pathways are at least partially conserved. In vascular plants, DELLA proteins play an important role in regulation of GA homeostasis, and serve as integrators of hormonal and environmental signaling pathways that constrain growth under adverse conditions (Davière et al. 2008). In *Arabidopsis* and most conifers, the less-polar GA_4 serves as a mobile, florigenic signal, rapidly accumulating at the meristem shortly before reproductive initiation (Odén et al. 1995; Eriksson et al. 2006; Fornara et al. 2010). In *Arabidopsis*, there is some overlap between the GA and age-related, vegetative phase change pathways, both of which regulate reproductive development (Yu et al. 2012). In both pathways, SPL transcription factors are released from suppression and activate FT and MADS box genes—notably the *SOC1* floral meristem identity gene—to trigger floral initiation (Moon et al. 2003; Achard et al. 2007; Yu et al. 2012). In the age-related pathway, aging reduces miR156 levels, reducing suppression of SPL. In the GA flowering pathway, GAs bind to GA INSENSITIVE DWARF1 (GID1) receptors, promoting degradation of DELLA proteins through the ubiquitin-proteasome pathway, and thus reducing DELLA-mediated transcriptional suppression of SPLs (Sun 2010; Yu et al. 2012). GA also independently regulates changes in morphological traits associated with miR156-mediated vegetative phase transition (Chien & Sussex 1996; Poethig 2003), which provides further evidence for crosstalk between GA and age-related pathways.

The GA-GID1-DELLA regulatory module of the GA signaling pathway is conserved in gymnosperms (Vandenbussche et al. 2007). Also, the ability of the meristem to respond to GA increases with age (Eysteinsson & Greenwood 1993; Greenwood et al. 2010), suggesting an overlap between the GA and phase change pathways, as in *Arabidopsis* (Poethig 2013). For example, juvenile conifers will not produce strobili even when high levels of endogenous GAs are present, nor consistently in response to exogenous application of GA (Zimmerman et al. 1985; Eysteinsson & Greenwood 1993). Likewise, very few strobili form on juvenile scions grafted into mature crowns, despite importation of GAs from neighboring branches (Greenwood et al. 2010; Day & Greenwood 2011). Thus GA regulation of strobilus formation is dependent on the state of the vegetative phase change pathway that governs the maturation of the meristem, just as is flowering in *Arabidopsis*. Since *FT* and *SOC1* orthologs have been identified in conifers, it is likely they are involved in GA-mediated reproductive development (Day &

Greenwood 2011). However, application of exogenous GAs to mature English ivy (*Hedera helix* L.) (Rogler & Dahmus 1974) and Australian blackwood (*Acacia melanoxylon* R. Br.) (Borchert 1965) results in production of morphologically juvenile leaves, indicating that GAs suppress vegetative phase change in some plants (Poethig 2013), and that underlying mechanisms are only partially conserved among spermatophytes.

2.3.4.2. Cytokinins (CKs)

Bud differentiation in conifers is partially regulated by CKs (Morris et al. 1990; Bollmark et al. 1995; Kong et al. 2012). Changes in CK biosynthesis and metabolism occur during maturation and often revert during reinvigoration, allowing ratios of various CKs to serve as phase change indicators in some species (Valdés et al. 2002; Valdés et al. 2004; Wendling et al. 2014a). Similar changes occur during bud initiation and differentiation (Kong et al. 2009), and differences have been noted between CK ratios in male and female buds (Kong et al. 2012). When applied after strobilus initiation, but prior to differentiation, exogenous CKs enhance female cone formation in Japanese red (*Pinus densiflora* Sieb. et Zucc.) and black (*P. thunbergii* Parl.) pine through conversion of male cones to female cones, possibly by changing the balance between CK and auxin in the developing bud (Wakushima 2004). Although these results have not been consistent across species, they suggest multiple roles for CK in reproductive development.

CKs appear to inhibit GA-mediated strobilus initiation, leading to the hypothesis that as a tree grows, the distance increases between the inducible meristem and the roots, where much of the CK is thought to originate (Smith & Greenwood 1995). This may produce a CK gradient that regulates both the timing of reproductive onset and the increase in reproductive potential as the tree ages (Greenwood et al. 2010). In support of this hypothesis, exogenous application of CKs prior to strobilus initiation reduces strobilus formation, whereas root pruning increases strobilus formation, possibly by limiting availability of root-produced CK (Smith & Greenwood 1995). In *Arabidopsis*, however, flowering time is not affected in transgenic plants lacking roots, indicating that roots have no significant role in phase change (Yang et al. 2011). In common pea (*Pisum sativum* L.), removal of the apical bud results in local CK synthesis in the stem, suggesting that auxins downregulate CK biosynthesis in the stem (Tanaka et al. 2006). In Nordmann fir (*Abies nordmanniana* [Steven] Spach), large differences in CK profiles in adjacent buds suggest local CK synthesis, and CK profiles correlate with different bud fates, suggesting a role for CK in bud-fate determination (Rasmussen et al. 2009). Also, CK levels in the bud and stem decrease following bud break, reaching a minimum just as shoot expansion reaches a maximum, then increasing as shoot elongation slows. This might be due to auxin-mediated regulation of local CK biosynthesis in the bud and stem. Taken together, it appears that local CK biosynthesis may be more important than root-derived CKs in regulating reproduction. The mechanism by which root-pruning enhances reproductive output remains unclear; it may be through changes in ratios of CKs, a reduction in total CKs, or through another pathway, possibly the miR156/SPL regulatory module implicated in stress response (Stief et al. 2014).

2.3.4.3. Auxins

Auxins are central to plant growth and development (Curaba et al. 2014), so it would not be surprising to find a role for them in phase change and reproductive development in conifers. Auxins, together with CKs and GAs, regulate the mitotic activity that maintains the state of maturation of the meristem (Haffner et al. 1991; Valdés et al. 2002). Concentrations of the endogenous auxin indole-3-acetic acid (IAA) increase during the period of active, vertical growth during juvenility, and are then maintained or decrease during the adult vegetative and reproductive phases (Valdés et al. 2002), coincident with declining vigor and changes in branching habit (Greenwood et al. 2010). High concentrations of IAA during shoot elongation—which spans reproductive bud initiation and differentiation—correlate with heavy megastrobilus production in highly productive genotypes of Douglas fir, but no trend is observed in less productive genotypes, suggesting that high concentrations of IAA upregulate megastrobilus production (Kong et al. 2009). Similarly, IAA concentrations are higher in adult than juvenile buds and needles in several species (Andrés et al. 2002; Valdés et al. 2002; Valdés, Centeno, et al. 2003), but not all (Valdés et al. 2004).

Auxins may also play a role in gender determination. Application of exogenous auxin increased microstrobilus and decreased megastrobilus formation in *Picea*, *Pinus* and *Larix* spp., although results were inconsistent (Sheng & Wang 1990). During the period of microstrobilus formation in lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm), concentrations of IAA are higher in proximal regions of shoots, where microstrobili form, than in distal regions, where megastrobili will subsequently develop (Kong et al. 2012). This may suggest local production of auxin (Zhao 2008), or that developing male cone buds are particularly strong sinks for apically-produced auxin.

2.3.4.4. Abscisic acid

ABA levels increase from the juvenile to reproductive phase, and decrease during reinvigoration, indicating that phase transition is partially regulated by ABA in conifers (Haffner et al. 1991; Valdés et al. 2002; Valdés, Fernández, et al. 2003; Valdés et al. 2004; Materán et al. 2009). After reproductive onset, the role of ABA is less clear (Valdés et al. 2004), and ABA levels may plateau or even decline (Munné-Bosch 2007). In addition to the general upward trend during early maturation, ABA levels are fine-tuned in response to environmental stress signals—including extreme temperature, drought, and physical wounding (Tuteja 2007)—thus serving to integrate environmental cues into developmental pathways, possibly through interaction with the GA-DELLA or miR156/*SPL* module.

2.3.4.5. Ethylene

Local ethylene biosynthesis is rapidly upregulated in response to stress (Davies 2010). For example, ethylene mediates the response of plants to mechanical perturbation, including wind stress and physical wounding, resulting in increased radial growth and decreased elongation growth (Biro & Jaffe 1984; Telewski & Jaffe 1986). In an apple (*Malus domestica* Borkh.) hybrid seedling (Jonathan × Golden Delicious), an ethylene response factor (37-416_J) is upregulated during the juvenile phase, suggesting possible heighted sensitivity to environmental stimuli (Gao et al. 2013), and application of BAP (6-Benzylaminopurine, a synthetic CK) + ethephon (an ethylene-releasing compound) results in precocious flowering (Zhang et al. 2007). Ethylene has also been implicated in regulation of photoperiodic flowering, but effects are species-specific (Thomas & Vince-Prue 1997; Davies 2010). These results suggest that ethylene mediates developmental responsiveness to stress, probably through its interaction with the GA-DELLA module (Achard et al. 2007) or the miR156/*SPL* module.

2.4. EXOGENOUS REGULATION

2.4.1. Tree size

Although reproduction is more a function of meristem maturation than tree size (Greenwood et al. 2010), there is generally a positive relationship between tree size and reproductive output. This relationship is weakened by genetic variability and plasticity in response to environmental conditions, but remains significant (Haymes & Fox 2012; Santos-Del-

Blanco et al. 2013). Reproductive onset is also related to size, with a genetically-determined critical size required before reproduction may commence in many plants (Roff 2000), including apple and pear (Hackett 1985). This is supported by grafting studies in conifers: Few cones are produced by mature scions in the first few years (Greenwood et al. 1989; Day & Greenwood 2011), suggesting that the minimum size for reproduction has not been reached (Day & Greenwood 2011).

There exists an allocational tradeoff between reproduction and vegetative growth: For many trees, reproductive output is reduced and reproduction is delayed under conditions favorable to vegetative growth (Day & Greenwood 2011; Santos-Del-Blanco et al. 2013). In red spruce, for example, irrigation increases vegetative growth while simultaneously reducing cone production (Day & Greenwood 2011). This is particularly true for trees which have reached reproductive onset, but not reproductive competence, which will not generally reproduce except in the presence of sufficiently strong environmental signals (Williams 2009).

However, the time to reproductive onset may be shortened in environments that encourage rapid growth, such as under controlled greenhouse conditions, presumably because the size threshold is reached earlier. Similarly, time to reproductive onset may be extended by conditions that repress growth, such as low temperature or lack of resources (e.g., nutrients, water, light) (Hackett 1985; Poethig 1990; Bond et al. 2007; Amasino & Michaels 2010; Turnbull 2011). However, once the size threshold has been reached, those same stress conditions may encourage reproductive precocity and fecundity, resulting in early and heavy reproduction in smaller trees (Santos-Del-Blanco et al. 2013). Other factors, such as status in the canopy and competition, are also important, resulting in cone production that is highly variable and difficult to predict by size alone (Haymes & Fox 2012).

2.4.2. Environmental factors

2.4.2.1. Temperature

Hot, dry summers increase reproductive output for many forest trees (Owens & Blake 1985). Correlation studies have found significant positive relationships between cone or seed production and temperature during the summer of strobilus initiation for many species, such as Norway spruce (Solberg 2004), western hemlock (*Tsuga heterophylla* [Raf.] Sarg.) (Pollard & Portlock 1984), Scots pine (Ozolincius and Sujetoviene in Ozolinčius et al. 2009), balsam fir, and white spruce (*Picea glauca* [Moench] Voss) (Messaoud et al. 2007). No correlation was found for black spruce (*Picea mariana* [Miller] B.S.P.), which yields heavier and more frequent cone crops independent of climate conditions (Messaoud et al. 2007). Increased precipitation and cooler temperatures during all other years correlates with higher seed production in white spruce. This suggests that resource accumulation and allocation is critical during the years prior to cone initiation, and likewise during the year following as seeds develop, but that hot, dry conditions are required to trigger strobilus initiation (Roland et al. 2014).

2.4.2.2. Moisture

The results of correlation studies in Norway spruce suggest that high temperatures during cone initiation are a stronger determinant of cone production than is precipitation, yet precipitation remains a significant factor (Solberg 2004). Drought stress reduces soil water potential, making it more difficult for a plant to take up water and nutrients, which alters the physiology and reduces growth (Smith & Greenwood 1997; Sardans et al. 2008). Under moderate or even severe drought conditions, reproductive development may be enhanced, as an allocation tradeoff with vegetative growth (Owens & Blake 1985; Muller-Starck & Seifert 2008; Ozolinčius et al. 2009). However, under severe, prolonged drought, generative growth may be reduced. In Scots pine, reproductive bud initiation occurs two years prior to cone emergence. However, under multi-year, induced drought conditions, reproductive output dropped dramatically two years after the treatment was set in place—concurrent with 40% defoliation—and remained suppressed until the canopy had recovered, three years after removal of the water-excluding, sub-canopy roofs (Ozolinčius et al. 2009). It is possible that drought had enhanced cone bud initiation in the first year, but that bud survival declined under prolonged drought (cf. Ebell 1971). Cones initiated the first year of drought may have been aborted in the second year as limited resources were allocated to the growth of existing cones. Note that in the second year of drought, cone production was equal to that of control trees, despite significant defoliation, indicating a tradeoff in resource allocation.

2.4.2.3. Light

Although temperature is the more important factor driving cone production across larger scales, higher light interception locally increases cone production. Whereas temperate conifers on southern slopes (higher irradiance) tend to produce the most cones (Despland & Houle 1997), shaded trees may produce no cones (Chałupka & Giertych 1977). Likewise, for a given tree, the section of the crown receiving the most irradiance yields the most cones (Despland & Houle 1997). Light interception explains part of the variation in cone production based on canopy status. For example, a white spruce tree will produce cones in a plantation in full sun once it is 3 m high, but must be 14 m high to produce cones as a subcanopy tree (Greene et al. 2002). , Scots pine trees produce much heavier cone crops subsequent to release-thinning, and the majority of cones form further down the tree, in areas now exposed to light (Karlsson 2000). Experimentally, female strobili may be induced in Scots pine and Norway spruce by channeling natural light directly into the apical dome via optical fibers (Kosiński & Giertych 1982).

2.4.2.4. Photoperiod

In north temperate species, photoperiod regulates growth cessation, bud set, and entry into dormancy (Ekberg et al. 1979). Photoperiod is an important regulator of reproductive development, because the timing of strobilus initiation and differentiation is closely tied to shoot phenology (Owens & Blake 1985). In *Picea*, *Pinus*, *Abies*, and *Larix*, the maximum rate of growth corresponds to maximum day length, not temperature, indicating photoperiodic control of vegetative growth (Rossi et al. 2006). In *Pinus* spp., female strobili differentiate only 2 to 3 weeks prior to male strobili, yet seem to have different photoperiod requirements, suggesting photoperiod regulation of sex-expression (Giertych 1967). Experimental manipulation of photoperiod demonstrates the role of photoperiod in gender determination in western hemlock: An 18 h photoperiod during cone bud initiation increases male strobili, whereas a 13 h photoperiod increases female strobili (Pollard & Portlock 1984).

2.4.2.5. Nutrition

Cone production generally correlates positively with nutrition (Owens and Blake, 1985; Rothstein and Cregg, 2005; Owens et al., 2001). Phosphorus—and to a lesser extent nitrogen—was found to limit cone production in Fraser fir (*Abies fraseri* [Pursh] Poir.), when comparing populations with high and low reproductive output (Arnold et al. 1992). In ponderosa pine, cone production increased linearly in response to increasing rates of urea ammonium phosphate (Heidmann 1984). The linear response suggests that fertilization may enhance cone production when nutrients are limiting.

2.5. MANAGING CONE INDUCTION

Early efforts to enhance seed production in seed orchards relied on cultural treatments, such as fertilization, girdling, root pruning, and drought (Puritch 1972). Results were inconsistent, and occasionally detrimental (Puritch 1979). This is due in part to inter-species variability, and in part to the complex nature of regulation of maturation and reproductive development in conifers. Subsequent work with plant growth regulators (PGRs)—particularly exogenous application of GAs—resulted in greatly enhanced cone production, especially when combined with cultural treatments, although results varied by species, application method and timing, and environmental conditions (Puritch 1979). In a few species, juvenility could be overcome by application of GAs, resulting in precocious cone formation (Chałupka 1991), but results were generally temporary (Longman 1987). Juvenility and environmental conditions remain the primary factors that constrain our ability to induce cone development (Bonnet-Masimbert & Webber 1995). The most common treatments used to enhance cone production are briefly reviewed here, but have been extensively reviewed elsewhere (see Owens & Blake 1985; Ross & Pharis 1985; Bonnet-Masimbert & Webber 1995)

Because many cone enhancement techniques stress the tree and heavy cone yields are strong sinks for photosynthates (Dickmann & Kozlowski 1968; Dickmann & Kozlowski 1970), use of enhancing techniques on very young trees may have a detrimental effect on long term reproductive output (Ebell 1971; Roff 2000; Obeso 2002). Rather, practices that promote rapid vegetative growth in young trees will help them to attain reproductive size more quickly (Hackett 1985), after which time cone enhancement techniques may be applied, but must be balanced against the long term health of the tree.

2.5.1. PGRs

Application of GAs is the most broadly effective way to promote strobilus induction in temperate conifers. Efficacy is affected by many factors, including product quality, application rate and timing; and size, physiological age, and genotype of the tree. GA application is much more effective when combined with stress treatments, such as girdling, tenting, or water exclusion. Treatments must be applied during the period of strobilus initiation and differentiation to be effective (Bonnet-Masimbert & Zaerr 1987; Pharis et al. 1987; Owens 1995; Owens et al. 2001). In Cupressaceae and Taxodiaceae, GA_3 is the most effective (Longman et al. 1982). In Pinaceae, the less polar GAs, GA_4 and GA_7, are most effective (Owens & Blake 1985). GA is commonly applied by stem injection or foliar spray, beginning shortly after vegetative bud-break, and reapplied several times until strobilus initiation is complete (Owens & Blake 1985; Bonnet-Masimbert 1987; Funda & El-Kassaby 2013). Although exogenous GA application has been shown to promote strobilus formation in a few juvenile conifers (Chałupka 1991), and the inductive effects of GA application may carry over into subsequent years (Johnsen et al. 1994), exogenous application of GA does not promote phase change: Juvenile trees remain juvenile (Zimmerman et al. 1985).

The effects of cytokinins on cone formation are species specific. Exogenous application of the synthetic cytokinin 6-Benzylaminopurine (BA) enhanced female cone formation in Japanese red and black pines (Wakushima 2004), but had the opposite effect in black spruce (Smith & Greenwood 1995) and Chinese pine (*Pinus tabuliformis* Carr.) (Sheng & Wang 1990). GA + BA enhanced both male and female strobili production in Douglas fir (Ross & Pharis 1976) and Sitka spurce (*Picea sitchensis* [Bong.] Carr.) (Tompsett 1977).

Other PGRs may act synergistically with GA to enhance cone formation, but results are species specific and often variable (Bonnet-Masimbert 1987), perhaps due to differences in product quality or application rate and timing. In combination with GA, the synthetic auxin 1-Naphthaleneacetic acid (NAA) may enhance female but not male flowering in some species, such as lodgepole pine (Wheeler et al. 1980). In a few species, such as Sitka spurce (Tompsett

1977) and Douglas fir (Ross 1975), GA + NAA application promotes male strobilus formation at the expense of female development, although low rates of NAA may promote female strobilus formation in Douglas fir (Pharis et al. 1980). Similarly, chlorocholine chloride (CCC), a GA biosynthesis inhibitor, promotes male strobilus formation at high rates and female strobili at lower rates in Chinese pine, but the female strobili do not persist to maturity (Zhao et al. 2011). Application of paclobutrazol, another GA biosynthesis inhibitor, results in precocious, profuse cone formation in *Eucalyptus* (Griffin et al. 1993; Williams et al. 2003). Ethephon (metabolizes to ethylene) enhances female strobilus formation in Norway spruce when applied alone (Remrod in Bonnet-Masimbert 1989). Ethephon enhances both male and female strobilus production in Cupressaceae, but only when applied in combination with GA_3 (Bonnet-Masimbert 1971; Hashizume 1975).

2.5.2. Cultural treatments

Cultural treatments—such as girdling, root-pruning, fertilization, heat, and drought—are frequently used in seed orchards as an adjunct to GA's to synergistically enhance cone production, particularly in young conifers, or when weather conditions during bud initiation are not conducive to cone development. Cone enhancement may occasionally be obtained by application of a single cultural treatment, but most work better in conjunction with other cultural treatments, and often no treatment effects are observed unless GA is also applied (Owens & Blake 1985; Bonnet-Masimbert 1987; Pharis et al. 1987). A few of the more common cultural techniques are briefly reviewed here.

2.5.2.1. Fertilization

Nitrogen fertilizer is often applied to increase cone production in conifers, but results are highly variable (Owens & Blake 1985; Bonnet-Masimbert & Webber 1995; Miller & DeBell

2013), and addition of nitrogen alone may sometimes increase vegetative growth at the expense of generative growth (Krannitz & Duralia 2004). In general, cone production is positively correlated with nutrition (Owens & Blake 1985; Owens et al. 2001; Rothstein & Cregg 2005). In Pacific silver fir (*Abies amabilis* [Douglas ex Loudon] Douglas ex Forbes), the combination of fertilization, $GA_{4/7}$, and girdling promotes strobilus formation, but fertilizer alone was not tested (Owens et al. 2001). In *Eucalyptus nitens*, nitrogen fertilizer promotes strobilus development, but phosphorus fertilizer has no effect. Nitrogen fertilization results in increased tree size, which partially accounts for the increase in reproductive output (Williams et al. 2003). In Douglas fir, application of nitrate nitrogen increases cone production, but application of ammonium nitrogen does not (Stoate et al. 1961; Ebell 1966). There are no differences in foliar nitrogen content, bud density or growth, indicating that the reproductive response is independent of any growth response resulting from increased nitrogen uptake. Nitrate nitrogen fertilization results in changes in nitrogen metabolism that increase arginine and other free amino acid levels, with a corresponding increase in lateral bud survival during shoot elongation. Thus, more buds are available to develop into strobili (Ebell 1972a). Whether nitrogen fertilization acts in other ways to promote strobilus initiation or development is not known (Bonnet-Masimbert & Webber 1995). Unfortunately, even in Douglas fir results are not consistent across sites (Ebell 1972a; Miller & DeBell 2013), and a similar stimulatory effect of fertilization on reproductive output has not been reliably demonstrated in most conifers (Owens & Blake 1985).

2.5.2.2. Girdling

Of the various cultural practices tested, stem girdling is one of most effective at stimulating strobilus production in conifers (Wheeler & Masters 1985). Girdling refers to the removal of a thin strip of bark around the circumference of a stem, and results in accumulation of photosynthates above the girdle due to interruption of phloem transport. A common technique is to remove a strip of bark (3 – 30 mm wide) from just over half the circumference of the trunk, and a second, partially overlapping strip from higher up (e.g., 70 cm) on the other side of the tree. This effectively disrupts the phloem while reducing long term detrimental effects (Ebell 1971; Wheeler & Masters 1985). Even so, repeated girdling over many years may damage tree health and decrease reproductive output (Owens & Blake 1985). Strangulation (banding) is similar to girdling, but is generally less effective at cone enhancement. It involves the tightening of a restrictive band around the phloem to disrupt translocation (Owens & Blake 1985).

Girdling alone increases cone production in Douglas fir (Ebell 1971; Wheeler & Masters 1985), but girdling is more effective in combination with GA (Owens & Blake 1985; Kolpak et al. 2014). In Douglas fir trees of seedling origin that had not yet reached reproductive competence, girdling alone had no effect, but girdling combined with low levels of applied $GA_{4/7}$ did (Munoz-Gutierrez et al. 2010). Girdling is generally applied about 1 month prior to bud break, although optimal timing varies based on site-specific factors (Miller & DeBell 2013). The inductive effects may persist for several years. Cone production may be reduced if girdling is applied after the first week subsequent to bud break (Ebell 1971).

Girdling does not generally promote cone formation in most species unless used in combination with GA (Ross & Greenwood 1979; Miller & DeBell 2013). In ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.), girdling alone increases cone production, with no carry over effect to subsequent years (Shearer & Schmidt 1970). In western larch, (*Larix occidentalis* Nutt.), girdling alone increases cone development during a low cone production year, but not during a high cone production year (Chałupka 2008), suggesting an interaction between multiple signaling pathways.

The mechanism by which girdling acts to enhance cone production is unclear. In Douglas fir, girdling does not change the number of buds initially formed, but does increase bud survival (Ebell 1971), identical to nitrate fertilization (Ebell 1972a). However, reproductive bud survival did not correlate with carbohydrate levels, suggesting that disruption of phloem translocation is not the driver of bud survival in girdled trees (Ebell 1971).

2.5.2.3. Root pruning and transplanting

Although root pruning alone is occasionally effective in cone enhancement (e.g., Marquard & Hanover 1984; Webber et al. 1985), it is more commonly used to synergistically increase the responsiveness of a tree to GA treatments, and is most effective when applied near the time of vegetative bud break (Smith & Greenwood 1995). Although root pruning reduces the surface area of the root that is available for water absorption, it seems unlikely that cone induction results from a drought-response mechanism, since predawn water potential decreases in response to drought but may be unaffected by root pruning treatments (Smith & Greenwood 1997). Root pruning may act by altering hormone ratios or reducing the concentration of an inhibitory substance produced by the roots. Since application of CK can reduce cone formation and offset the effects of exogenous GA, root pruning may act by altering the GA to CK ratio (Smith & Greenwood 1995; Smith & Greenwood 1997; Greenwood et al. 2010). Transplanting has effects similar to root pruning, presumably by the same mechanism (Owens & Blake 1985).

2.5.2.4. Heat

Heat treatment alone, often implemented by placing the tree under polyethylene to trap solar heat, results in increases in strobilus production for some conifers, such as Sitka spruce (Tompsett 1977). Timing is critical. In Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), heat represses cone production when applied during early, rapid shoot elongation, but enhances cone production when applied toward the end of lateral shoot elongation, when growth has slowed and lateral bud determination occurs. The treatment window to promote female strobili is several weeks later than that for male strobili, which develop on lower, less vigorous branches that complete growth sooner (Ross 1985). This may explain why heat alone increased male strobilus production, but had no effect on female strobilus production, in Norway spruce (Chałupka & Giertych 1977; Johnsen et al. 1994). Note that when combined with GA, timing is less critical, and heat increases both male and female cone production in many species, including *Picea* spp. (Philipson 1992; Johnsen et al. 1994), western hemlock (Pollard & Portlock 1981), and Pacific silver fir (Owens et al. 2001). In Scots pine, however, solar heating suppresses female cone production, alone and in combination with GA (Chałupka 1981). In *Picea* spp., the effects of heat and GA are only additive (Tompsett 1977; Chałupka 1981), suggesting a common mechanism for cone enhancement. Indeed, the levels of less-polar GA's believed to play a role in generative determination increase after 1 day of heat treatment in Norway spruce, and remain elevated for 2 to 3 weeks (Chałupka et al. 1982). This may explain why heat treatment is only effective in enhancing female cone production when applied during late growth, precisely at the time of strobilus initiation. When applied early in the season, the elevated levels of endogenous GA's may return to normal levels prior to generative determination.

2.5.2.5. Drought

Drought alone is sufficient to enhance cone production in some conifers, such as Engelmann spruce (Ross 1985), Douglas fir (Ebell 1967 in Puritch 1972), and jack pine (*Pinus banksiana* Lamb.) (Riemenschneider 1985). Timing is critical, and varies by species. Spring irrigation followed by summer drought increases female strobilus production in loblolly pine (Dewers and Moehring Puritch 1972). In Engelmann spruce, the timing of inductive drought is opposite to that of heat: Drought enhances cone production when applied during early, rapid shoot elongation, but represses cone production when applied toward the end of lateral shoot elongation, when growth has slowed and lateral bud determination occurs (Ross 1985).

Drought is more commonly applied together with GA and other cultural treatments, such as heat or girdling (Owens & Blake 1985; Smith & Greenwood 1997). In Sitka spruce, drought + heat + GA resulted in increased production of male and female cones during a cool, wet summer, when GA alone had no treatment effect (Philipson 1992). In Douglas fir, inductive drought increases levels of arginine and other amino acids (Ebell & McMullan 1970), which might suggest a common mechanism with nitrate fertilization (Ebell 1972b).

2.5.2.6. Possible mechanisms

When cultural practices are combined with exogenous GAs, the effect on cone enhancement is often synergistic, suggesting multiple reproductive pathways (Pharis et al. 1987). When cultural practices are used alone, effects are generally much less dramatic or non-existent. This supports the hypothesis that there are multiple pathways that must each create a permissive state for reproduction to occur (Ross & Pharis 1985). However, environmental factors, such as photoperiod, temperature, and nitrogen availability, regulate GA biosynthesis and metabolism, suggesting a possible common mechanism for environmental control of reproductive development in conifers (Chałupka et al. 1982; Pollard & Portlock 1984; Odén et al. 1995). Indeed, many cultural treatments that enhance cone production—including drought, girdling, heat, root-pruning, and nitrate fertilization—alter the levels of GAs, frequently increasing the levels of the less polar, inductive GAs, and decreasing the levels of the more polar GAs. These changes in GA biosynthesis and metabolism may in part explain the promotive effect of such treatments (Pharis et al. 1987; Bonnet-Masimbert 1989). Other changes occur in the levels of

ABA, CK, and ethylene in response to certain cultural treatments, so crosstalk between multiple hormonal signaling pathways may be involved in regulating reproduction in response to such treatments (Bonnet-Masimbert 1989).

Environmental and stress signaling also regulate the levels of many microRNAs—notably miR156 (Hsieh et al. 2009; Lee et al. 2010; Sun et al. 2012; Kim et al. 2012; Stief et al. 2014). Since miR156 regulates phase change, cultural treatments may indirectly act to prolong or shorten juvenility by altering miR156 levels (Yamaguchi & Abe 2012).

2.5.3. Controlling ratio of male to female cones

Spatial separation of male and female cones into zones within the crown is common among conifers (Ross & Pharis 1987). In some conifers, such as *Pinus* and *Tsuga* spp., development of male and female cones is separated both temporally (female cones differentiate a few weeks after male cones) and spatially (Owens & Blake 1985). Thus, male strobili develop under and may require slightly different environmental conditions (longer photoperiod, less direct light, different temperature) than do female strobili (Giertych 1967). Since GA acts in both male and female strobilus development, GA application can be timed to preferentially enhance one sex over the other in species that display temporal separation of male and female bud differentiation (Chałupka 1981). Manipulation of photoperiod can influence sex expression in some species. In western hemlock, for example, an 18 h photoperiod favors male strobili, and a 13 h photoperiod favors female development (Pollard et al., 1984).

Apical meristems exhibit considerable sexual plasticity during differentiation, as evidenced by the occasional formation of bisporangiate cones (containing both micro- and mega-sporophylls) in nearly all conifer species (Steil 1918; Holmes 1932; Ross & Pharis 1987). It is therefore possible to manipulate sex expression in some species by altering the environmental conditions or hormone levels during differentiation to favor one sex over the other (Ross & Pharis 1987). For example, auxin may be involved in male strobilus development. IAA is maintained at higher concentrations in proximal areas, where male cones form, during the period of male cone bud differentiation in lodgepole pine (Kong et al. 2012). Auxin applied alone stimulates male and reduces female cone production in some species, such as Chinese pine (Sheng & Wang 1990).

CKs also appear to have a role in sex determination in some conifers, and changes in CK biosynthesis and metabolism have been implicated in sex determination (Kong et al. 2012). When applied prior to strobilus initiation, CK inhibits GA-induced strobilus initiation. When applied after male strobilus initiation, but prior to differentiation, CKs enhance female cone formation in Japanese pines through conversion of male cones to female cones (Wakushima 2004). Thus, the ratio of CK to auxin may be important, with auxins favoring male development and CKs favoring female development. It is likely that other hormones, such as ABA (Kong et al. 2012) and ethylene (Ross & Pharis 1987), play some role in sex determination.

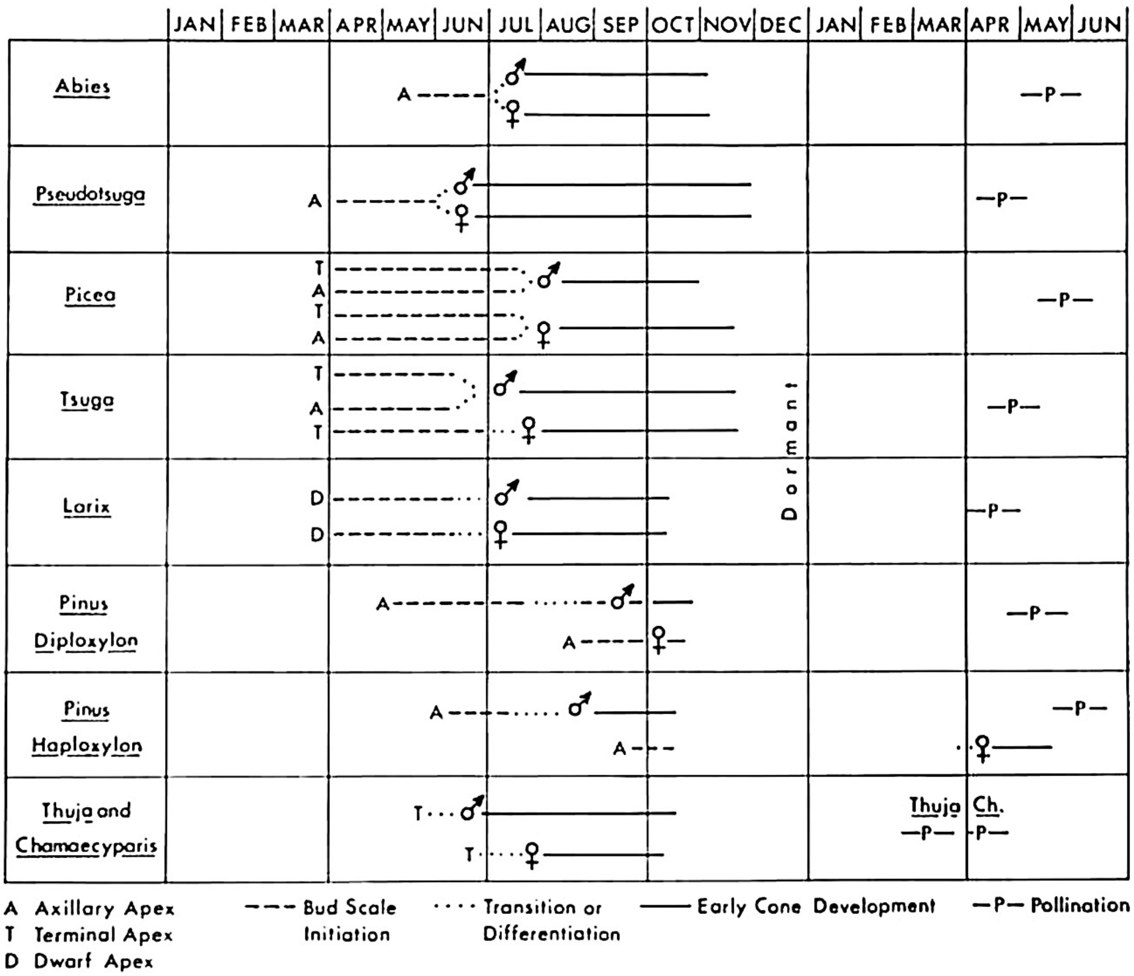
There is considerable indirect evidence to support a role for vigor in sex expression, with female cone production associated with sites of high vigor, and male cone production associated with sites of lower vigor. These effects appear to be indirect, with sex determination mediated through changes in hormone signaling. Nevertheless, treatments that affect vigor may be useful in regulating sex determination (Ross & Pharis 1987).

2.5.4. Seed orchard panmixia

Most seed orchards are designed to encourage panmictic equillibrium, meaning that each clone or family is equally likely to mate with any other clone or family, ensuring equal contribution to the genetic diversity of the progeny (Funda & El-Kassaby 2013). However, there is considerable variation in fecundity and reproductive phenology among clones, families, and individuals, making such orchard harmonization difficult (El-Kassaby et al. 1989; El-Kassaby & Askew 1991). Half of the parentage in a seed orchard may be attributed to just 20% of the trees (Funda 2012), and less than half of the trees in a seed orchard may contribute meaningfully to genetic diversity (Chałupka 2008). Cone enhancement techniques may be used on individual trees to improve panmixia. Also, because cone enhancement techniques often have a greater effect on less productive trees, they may improve genetic contribution even when applied broadly across an orchard (Chałupka 2008). Techniques that preferentially promote the formation of cones of one sex over the other may be useful to correct problems with sexual asymmetry in species with unequal male and female fertility (Choi et al. 2004; Codesido & Fernández-López 2013). Mist cooling may be used to delay bud break and compress reproductive phenology, reducing background pollen contamination and improving synchrony within an orchard (Silen & Keane 1969; Funda & El-Kassaby 2013).

APPENDIX

Figure 2.1. Times and methods of cone initiation. From Forest tree seed production. 1985. Owens, J.N; Blake, M.D. Agriculture Canada, Can. For. Serv., Petawawa National Forestry Institute, Chalk River, Ont. Information Report PI-X-53. 161 p. Reproduced with permission, 2016.



CHAPTER THREE

PHENOLOGY MODEL IDENTIFIES TIMING OF CONE INITIATION IN FRASER FIR (*ABIES FRASERI*)

ABSTRACT

PHENOLOGY MODEL IDENTIFIES TIMING OF CONE INITIATION IN FRASER FIR (*ABIES FRASERI*)

Fraser fir (*Abies fraseri* [Pursh] Poir.) is an important Christmas tree species in the United States, but growers incur significant labor expenses in years with heavy cone production. Cultural practices or chemical treatments may limit formation of reproductive buds, but must coincide with the period of bud initiation and differentiation to be effective. Reproductive phenology is tied to lateral shoot phenology and governed by thermal time. We surveyed shoot phenology at nine Christmas tree plantations in Michigan over three years, and used the data obtained to develop a phenology model of shoot growth that predicts the timing of cone bud initiation and differentiation in growing degree days. The phenology model, based on the beta sigmoid function, offers high predictive power and is robust to extremes of temperature and precipitation. Our model provides guidance for timing practices that influence cone bud formation, both for reducing cones in Christmas tree plantations and alternatively for enhancing cone production in seed orchards. In addition, the model may be extended to assist with other practices tied to shoot phenology, such as the timing of pesticide applications or shearing.

3.1. INTRODUCTION

Fraser fir (*Abies fraseri* [Pursh] Poir.) is an important Christmas tree species in the United States, with annual sales of $100 million in the southern Appalachians (Pettersson et al. 2015), and a growing market share in the Midwest. Fraser fir trees grown in Christmas tree plantations often produce heavy cone crops, which is a significant problem for producers in the Midwest (Cregg et al. 2003), and increasingly in North Carolina (Owen 2015). Cones decrease the value of a tree by displacing lateral branches in the upper third of the tree crown, resulting in sparse tops that are less acceptable to consumers. Expanding cones also compete with vegetative growth for photosynthates (Powell 1977; Sala et al. 2012). Therefore, many growers remove cones by hand while they are still small. Per tree, cone removal represents the highest labor expense for many growers (Owen 2015).

In *Abies*, strobilus bud initiation and differentiation are closely tied to shoot phenology, and occur in early summer, the year before cones emerge. Cone buds initiate from lateral buds when growth of lateral shoots begins to slow. Differentiation of reproductive organs follows over the next two weeks, and is complete by the time lateral shoot expansion has ended (Owens & Blake 1985). Environmental signals during the period of initiation and differentiation interact with endogenous controls to regulate reproductive development (Owens & Blake 1985). Gibberellin (GA) signaling is involved in initiation of reproductive development (Pharis & Kuo 1977), and GA is commonly applied to induce or enhance cone production in seed orchards (Owens & Blake 1985; Ross & Pharis 1985; Philippe et al. 2004). High temperatures and drought increase cone production in many forest trees (Owens & Blake 1985; Solberg 2004), and techniques to increase tree stress, such as girdling or root pruning, are used in seed orchards to enhance cone production—generally in combination with GA (Ross & Pharis 1985; Smith & Greenwood 1997).

Two experimental approaches have been taken to mitigate the effects of heavy cone production in Fraser fir plantations. The first involves using sprays (chemical thinners or herbicides) to cause abortion of cones in the spring while they are still small (Cregg et al. 2003; Owen 2015). However, these products may cause significant needle phytotoxicity when applied at rates high enough to cause cone abortion. The second approach is to disrupt strobilus initiation or differentiation. This approach may allow more lateral buds to develop into shoots, resulting in more uniform branch density throughout the tree crown. Strobilus development may be disrupted by chemical treatments, such as application of plant growth regulators (PGRs) (Cregg et al. 2003; Crain & Cregg 2016a), and cultural practices that modify environmental conditions (Crain & Cregg 2016b). However, treatments need to precede reproductive bud initiation and continue throughout differentiation. PGR sprays, in particular, need to be carefully timed in order to be effective. For cone reduction, most relevant PGRs are GA inhibitors that interfere with gibberellin biosynthesis. Therefore, they might over-regulate stem elongation if applied too early, but may not affect cone production if applied too late. Because reproductive phenology is governed by thermal time, timing of PGR sprays or cultural treatments to reduce cone formation should be based on growing degree days (GDD), rather than calendar date.

The objective of this study was to build a model of shoot phenology in Fraser fir that predicts the timing of reproductive bud initiation and differentiation based on growing degree day accumulation.

3.2. MATERIALS AND METHODS

Our study spanned nine sites in Michigan and covered a period of three years (Fig. 3.1). Because of the number of cooperators involved, we selected leader growth for developing the phenology model because it can be easily and accurately measured with little specific training for grower-cooperators. However, reproductive bud development is tied to lateral shoot phenology, which advances ahead of terminal shoot phenology. Since both leader and lateral shoot elongation are governed by thermal time, lateral shoot growth should correlate with leader growth for incorporation into the phenology model. We divided the study into two phases, the first to develop the model and the second to incorporate lateral shoot phenology into the fitted model.

3.2.1. Phase I: Development of phenology model

From May 2011 – July 2012, we monitored terminal leader growth on Fraser fir trees in nine operational plantations representing a wide range of locations and site conditions in Michigan (Table 3.1). We randomly selected 25 trees at each site, and measured leader length each week beginning at bud break and ending when the average leader length for all trees measured in a field was unchanged from the previous week. We paired this growth data with GDD data (base 41°F, Baskerville-Emin method) obtained for each measurement date from nearby automated weather network stations operated by Michigan State University Enviro-weather (http://enviroweather.msu.edu).

We developed our phenology model from the measurements of leader growth. Because the graph of the response variable (leader length) against GDD followed a sigmoidal curve, we initially fit a logistic function, which is commonly used to model biological growth data (Yin et al. 2003). Despite the high R^2 (0.95), we noted bias in the graph of residuals against predicted values, indicating that the model did not accurately fit the data at the beginning and end of the growth curve. We then fit a beta sigmoid function (BSF), which is a more flexible, generalized polynomial equation that is able to accurately represent a variety of sigmoid plant growth patterns (Yin et al. 2003; Auzanneau et al. 2011; Shi et al. 2016), following the equation given by Shi et al. (2016):

$$l = c_m(t - t_b)\left(\frac{2t_e - t_m - t}{2t_e - t_m - t_b}\right)\left(\frac{t - t_b}{t_m - t_b}\right)^{\left(\frac{t_m - t_b}{t_e - t_m}\right)}$$

where $t_b \leq t \leq t_e$, l is shoot length as percent of maximum length, t is thermal time (°Cd), and c_m is the maximum growth rate occurring at time t_m. This model is simplified to assume that growth rate is 0 at the beginning (t_b) and end (t_e) of the growth period.

We fit the model in R 3.2.4 (R Core Team 2016) using functions developed by Shi et al. (2016). We trained the model on growth data from 2011 collected at five sites, located an average of 6.9 mi. (11.1 km) from Enviro-weather automated network stations that supplied the GDD data (Fig. 3.1). Two data sets were used for external validation to test the predictive power of the model. The 2011 validation data set included growth data from 2011 collected at four study sites. For three sites, GDD data was calculated as an average of data from two or three Enviro-weather stations. For the fourth site, GDD data was obtained from a single weather station located nearby. The 2012 validation data set was primarily for temporal validation, and consisted of data from 2012, obtained from the five sites that contributed to the training data set and one site that contributed to the 2011 validation data set.

The phenology model was fitted and validated using the means of leader length data for each site and measurement date. To adjust for differences in vigor between sites, terminal leader length data were normalized as percent of maximum growth for each site by dividing the average leader length for each date by the average final leader length and multiplying by 100. Goodness of fit was assessed for the fitted phenology model using R^2, standard deviation error in calculation (SDEC), and visual assessment of residual plots (Frank & Todeschini 1994; Gramatica 2013). We assessed predictive power by separately fitting two validation data sets to the trained model and evaluating R^2, standard deviation error in prediction (SDEP) and residual plots for the fitted data. We calculated SDEC for the training set and SDEP for the validation sets by dividing the residual sum of squares (RSS) by the number of samples (n) and taking the square root.

3.2.2. Phase II: Mapping of lateral shoot phenology to model

To acquire lateral shoot data to incorporate into our phenology model, we tracked lateral shoot and leader growth from May 2012 – August 2013 at a subset of four sites randomly selected from among our phenology study sites (Fig. 3.1). Each week, we measured the length of the terminal leader and the length of one randomly selected, non-shaded, lateral shoot from the upper three whorls, south side of each of the 25 trees previously selected for inclusion in our phenology study. We began measurements at vegetative bud break and continued until average leader and lateral shoot length was unchanged from the previous week.

To incorporate lateral shoot phenology into our 2011 phenology model, we first fit the beta sigmoid function to the 2013 lateral shoot data, and then temporally validated that 2013 lateral shoot model using the 2012 lateral shoot data. From the trained and validated model, we derived (1) the time of maximum growth rate (T_{mLat}), after which reproductive bud initiation occurs, and (2) the time of lateral growth cessation (T_{eLat}), marking the end of reproductive bud anatomical differentiation. We then used T_{mLat} and T_{eLat} to map their corresponding shoot phenology parameters to the 2011 phenology model. We followed the same methods to fit the lateral shoot model as we used to fit the 2011 phenology model, including normalizing lateral growth data as percent maximum growth prior to fitting the model. The 2012 data from the Greenville site was excluded from the study because of inconsistencies in the data.

3.3. RESULTS

3.3.1. Phase I: Development of phenology model

The BSF model fit the leader growth data well, and provided high predictive power both spatially and temporally that was robust to high temperatures and drought (Table 3.2). SDEC or SDEP were below 0.04 (indicating fitting error ± 4.0%) and R^2 was 0.99 for both the trained model and the model fitted with the 2011 validation data set. Many sites experienced record warm weather in March 2012; nevertheless, the 2011 phenology model predicted the data well, with SDEP = 0.06 and R^2 = 0.98 for the fitted 2012 validation data set. We found no apparent bias in residual plots from fitted training or test data sets.

Model-fitting using the BSF results in parameters that are immediately biologically informative (Yin et al. 2003). Based on the fitted model, the period of active leader growth ranged from 480 – 2367°Fd (266 – 1315°Cd), with the growth rate peaking at 1421°Fd (789°Cd; Table 3.2; Fig. 3.2[a]).

3.3.2. Phase II: Mapping of lateral shoot phenology to model

Consistent with our field observations, we noted differences between model predictions of lateral shoot and terminal leader phenology. Bud break was slightly earlier (81°Fd [45°Cd]) for lateral shoots, which reached maximum growth rates 170°Fd (94°Cd) ahead of the leader. Growth rate was symmetrical for leader elongation, but growth rate of lateral shoots dropped rapidly after reaching its maximum. Lateral elongation was complete several days (512°Fd [285°Cd]) before leader elongation.

We obtained a good fit and good predictive power using the BSF to model lateral shoot elongation, with an R^2 of 0.97 and SDEC or SDEP of 0.06 for both the fitted 2013 lateral shoot model and for the 2012 validation data set fit to the 2013 model (Fig. 3.2[b]; Table 3.2). We identified key points in lateral shoot phenology to map to our 2011 phenology model (Fig. 3.3): bud break at 398°Fd (221°Cd), strobilus initiation shortly after 1250°Fd (695°Cd; T_{mLat}, the time of maximum growth rate), and completion of strobilus differentiation concurrent with growth cessation at 1854°Fd (1030°Cd). By plugging these parameters into our 2011 phenology model, we were able to express shoot phenology in terms of terminal leader phenology.

Strobilus initiation began around the time that terminal leader elongation was 35% complete, with differentiation complete once leader growth attained 80% of maximum length.

3.4. DISCUSSION

Using thermal time as the only factor, we developed a phenology model that is able to predict terminal leader and lateral shoot phenology in Fraser fir. The 2011 phenology model was developed and validated using data from nine sites that varied in climate and in site conditions. The predictive power of the model was high even during the extreme heat and drought conditions at the study locations in 2012. This suggests that the model is quite robust, and will predict Fraser fir shoot phenology with a high degree of accuracy for sites located throughout the Upper Midwest. However, the model will need to be validated with data from more distant sites before it may be applied with confidence in those locations, including the southern Appalachians.

Because reproductive phenology is tied to lateral shoot phenology in *Abies* (Owens & Blake 1985), our model is able to predict the window during which strobilus initiation and differentiation occur. This window may be used by Christmas tree producers, researchers, and seed orchard managers to manipulate reproductive development through use of treatments that modify hormonal (e.g., PGRs) or environmental signaling (e.g., temperature and water availability) pathways. These treatments must be applied prior to the period of strobilus initiation (around 1250°Fd [695°Cd], T_{mLat} in our phenology model) and continued until differentiation is complete (1854°Fd [1030°Cd], T_{eLat}).

The BSF produced a good fit to the shoot growth data and predicted shoot elongation well. However, in every case that we examined, the BSF slightly under-predicted the final shoot length as 99% of the observed maximum length, which may be due in part to underestimating

T_e. It is unclear whether this error is an artifact of the modeling process (due to the iterative nature of non-linear modeling), or a limitation of the BSF (see Shi et al. 2016). This problem of fit will not affect the utility of our model, but users of any model based on the BSF should be aware of this limitation. In the case of our phenology model, lateral shoot elongation—and therefore strobilus differentiation—may continue slightly past the time predicted by the model, T_{eLat}.

In addition to providing guidance for manipulation of cone production, our phenology model may assist Christmas tree producers in determining application rates and timing for certain pesticides. For examples, bud break in lateral shoots is an important phenological event predicted by our model that may be useful in timing sprays to control twig aphid. However, bud break is regulated by complex physiological processes that cannot be modeled solely on the basis of the GDD used in our phenology model (see García de Cortázar-Atauri et al. 2009). Therefore, bud break may vary by several days from the time predicted by our phenology model.

In conclusion, we developed a robust model to predict the timing of shoot phenology in Fraser fir. The 2011 phenology model predicts the window of strobilus initiation and differentiation, as well as the beginning and end of terminal leader and lateral shoot growth. This information may be used to time practices that reduce cone formation in Christmas tree plantations, or enhance cone production in seed orchards. Our phenology model may provide additional guidance to Christmas tree growers, such as indicating the window for application of pesticides and predicting when trees will be ready for shearing. The capabilities of this model may be easily extended through the addition of other important phenological events, such as those used to determine variable herbicide application rates throughout the growing season.

APPENDIX

Table 3.1. Site characteristics of study locations in Michigan, 2011.

	Coordinates (decimal degrees)		Weather station [a]	Soil [b]		Temperature [c] (°F)		Precipitation [d] (in.)	
City	Latitude	Longitude	(mi.)	Type (slope, %)	pH	Annual	June	Annual	June
Allegan	42.5475	-85.7796	18.8^2	Ockley loam (1 – 6)	5.8	52.5	68.3	36.5	4.2
Greenville	43.1806	-85.1394	11.0^2	Tekenink-Spinks loamy sands (6 – 12)	5.7	51.7	68.4	38.8	3.7
Hart†	43.7391	-86.2144	7.3	Spinks loamy fine sand (6 – 12)	6.2	48.5	64.4	34.7	3.2
Horton†	42.0760	-84.4830	19.7	Hillsdale-Riddles sandy loams (6 – 12)	6.2	10.5	19.8	80.0	8.3
Ida†	41.8846	-83.6237	5.1	Oakville fine sand (0 – 6)	6.5	50.9	68.5	35.3	3.6
Interlochen	44.5605	-85.7194	22.0^3	Karlin loamy sand (6 – 12)	5.8	48.0	63.9	33.1	3.2
Lapeer	42.9964	-83.3133	5.5	Miami loam (2 – 6)	6.1	49.9	66.8	32.5	3.4
Manton†	44.3986	-85.2875	0.3	Montcalm-Graycalm complex (0 – 6)	5.8	48.2	65.1	34.4	3.5
Mason†	42.6661	-84.4482	2.1	Capac loam (0 – 4)	6.6	50.8	67.6	32.2	3.5

a. Average distance from study plot to weather stations used to obtain growing degree data; superscript indicates number of stations used, if > 1.

b. Web soil survey (USDA NRCS).

c. Average daily temperature, 1981 – 2010 U.S. Climate Normals (NOAA 2010).

d. Total precipitation, 1981 – 2010 U.S. Climate Normals (NOAA 2010).

† Data were used to train 2011 phenology model. Other sites were used for validation.

Table 3.2. Fitted parameters and measures of fit and predictive power for phenology models of terminal leader growth and lateral growth in Fraser fir in Michigan Christmas tree plantations.

			SDEC or SDEP[a]	Fitted parameters [b]			
	n	R^2		C_m	T_b	T_e	T_m
2011 phenology model training	57	0.9889	0.0398	0.0785 (0.1414)	479.65 (266.42)	2366.69 (1314.78)	1420.66 (789.28)
2011 validation (spatial)	44	0.9930	0.0323				
2012 validation (temporal)	73	0.9775	0.0575				
2013 lateral shoot model training	36	0.9735	0.0601	0.1089 (0.1960)	398.40 (221.28)	1854.33 (1030.11)	1250.61 (694.83)
2012 validation (temporal)	30	0.9663	0.0556				

a. Standard deviation error in calculation or prediction. Low values indicate good fit to the data.

b. C_m is relative maximum growth rate (% max. growth rate/°Fd [°Cd]). T_b, T_e, and T_m are in °Fd (°Cd) and indicate the beginning of growth, the end of growth, and the time of maximum rate of growth, respectively.

Figure 3.1. Locations of Christmas tree farms that cooperated in the 2011 – 2013 phenology study in Michigan.

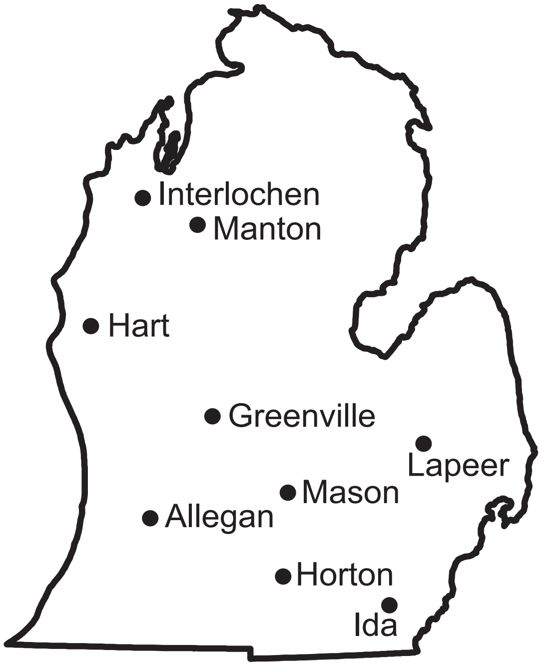


Figure 3.2. Fitted and validated (a) 2011 phenology model of leader growth and (b) 2013 phenology model of lateral shoot growth using a beta sigmoid function (BSF). The time of maximum growth rate is indicated by T_m for terminal leader growth or T_{mLat} for lateral shoot growth. Symbols indicating training or validation data represent mean growth of 25 trees.

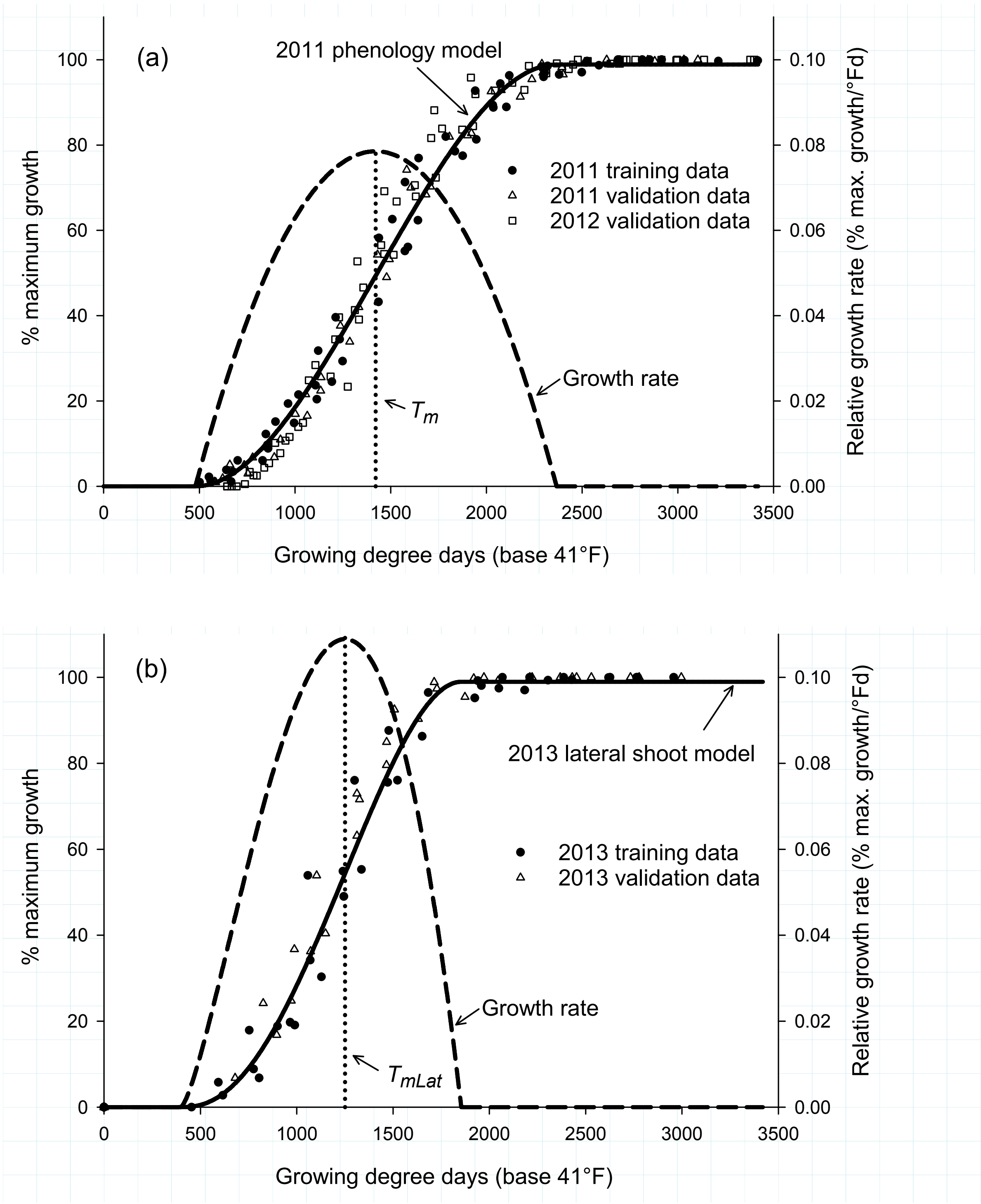
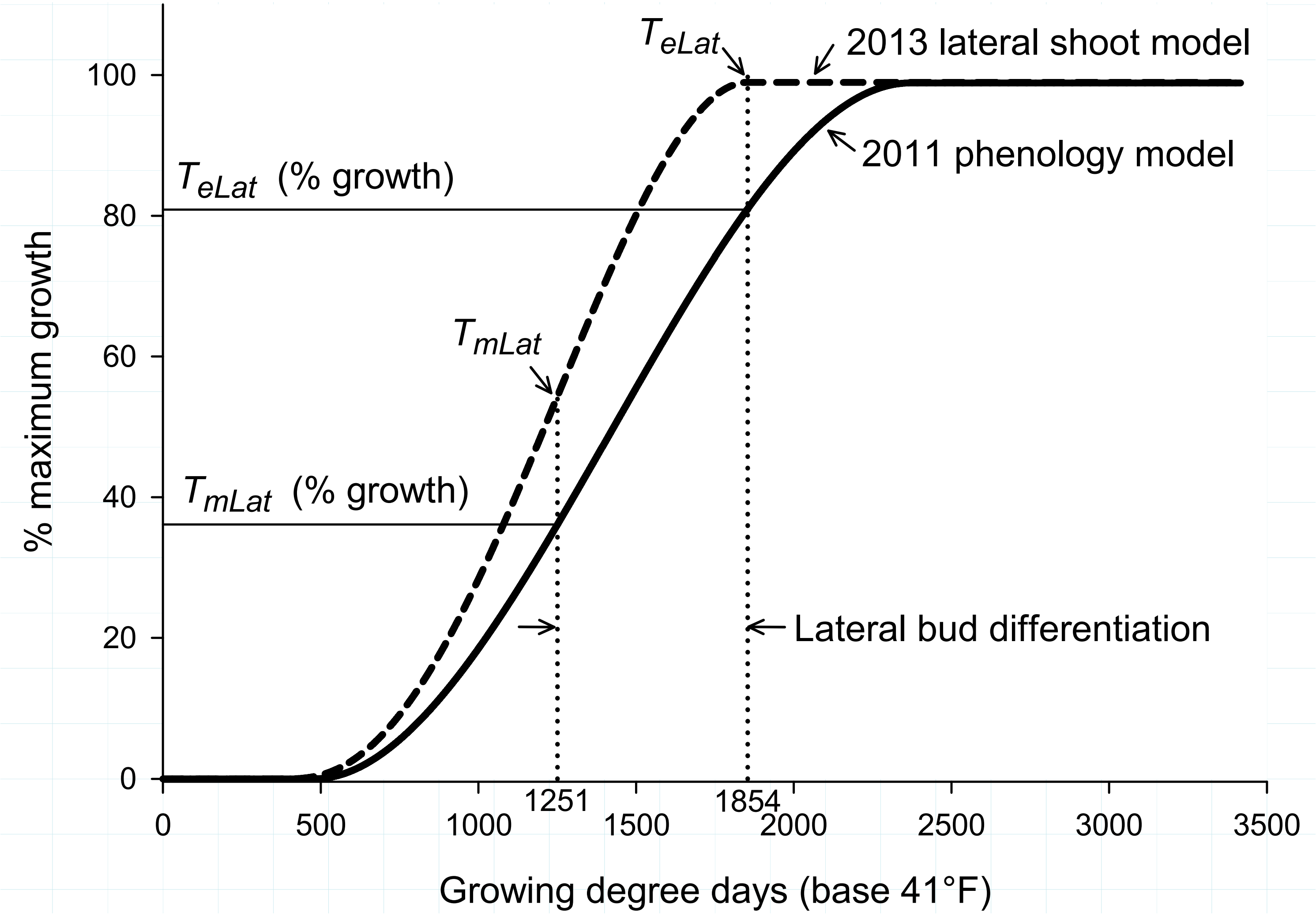


Figure 3.3. Mapping of Fraser fir lateral shoot phenology to 2011 phenology model of leader growth. T_{mLat} and T_{eLat} bound the period of strobilus bud initiation and differentiation, and indicate the time of maximum growth rate and the end of lateral shoot elongation, respectively.



CHAPTER FOUR

USING IRRIGATION AND MULCH TO CONTROL CONE PRODUCTION IN FRASER FIR (*ABIES FRASERI*)

ABSTRACT

USING IRRIGATION AND MULCH TO CONTROL CONE PRODUCTION IN FRASER FIR (*ABIES FRASERI*)

Fraser fir (*Abies fraseri*) is economically important as a Christmas tree in the United States, but trees often produce cones at a young age when grown in plantations in the Midwest. Early cone production is undesirable and decreases the economic value of the tree. High temperatures and drought are associated with increased reproductive output in conifers. Therefore, we investigated the effects of irrigation and mulch on cone production in two Fraser fir plantations in Michigan. At one site, we applied mulch to one tree in each row, with paired controls. In 2013, irrigation increased soil moisture by 47% percent under drought conditions, compared to non-irrigated control plots. However, irrigation had no effect on cone production in any year. Mulch reduced average maximum daily soil temperatures 7.4°C in 2013 and 4.8°C in 2014. Mulch reduced cone production in 2014 by 51%, but had no effect the following year, after a cooler summer. Our findings suggest that soil temperature, rather than soil moisture, regulates cone formation in Fraser fir, but treatments that reduce soil temperatures may only be effective in warmer locations or years.

4.1. INTRODUCTION

Michigan is the third largest producer of Christmas trees in the United States. In 2013, 1.7 million trees were harvested with a farm level value of $30.6 million, half of which came from sales of Fraser fir (*Abies fraseri* [Pursh] Poir.)(USDA NASS 2014). Fraser fir is considered a premium Christmas tree due to its beautiful form and color, strong branches, and exceptional needle retention. It was introduced from cool, moist temperate forests of the southern Appalachian Mountains, some 600 – 800 km south of Michigan. In natural stands, Fraser fir trees typically begin to produce cones when they are 15-20 years old (Johnson 1980). In Christmas tree plantations in Michigan and the upper Midwest, Fraser fir trees may produce cones just a few years after planting (Cregg et al. 2003). Developing cones displace lateral shoots, resulting in sparse tops that are undesirable to consumers. If left on the tree, cones compete for photosynthates, reducing vegetative growth, and then disintegrate in early fall, leaving behind the rachis (stalk) and resin-encrusted scales, further reducing the price that consumers are willing to pay for the tree. Therefore, many growers choose to remove the cones early in the growing season. Cone removal must be done by hand and represents the second largest labor expense for many growers, after shearing.

In temperate regions, reproductive phenology in seed plants is largely governed by thermal time. Once sufficient heat units (growing degree days [GDD]) have accumulated, reproductive bud initiation occurs (Hänninen 2016). In *Abies* spp., environmental factors interact with endogenous signals in early summer to regulate the initiation and differentiation of reproductive buds from lateral buds. Reproductive buds become dormant in the fall and develop into cones the following spring (Powell 1974; Owens & Blake 1985). High temperatures during strobilus initiation are correlated with increased cone production in many species, including Norway spruce (*Picea abies* L. Karst.)(Solberg 2004), western hemlock (*Tsuga heterophylla* [Raf.] Sarg.)(Pollard & Portlock 1984), Scots pine (*Pinus sylvestris* L.) (Ozolincius and Sujetoviene in Ozolinčius et al. 2009), balsam fir (*Abies balsamea* [L.] Mill.), and white spruce (*Picea glauca* [Moench] Voss)(Messaoud et al. 2007). Drought is also known to increase cone production in many forest trees (Owens & Blake 1985; Solberg 2004), as an allocation tradeoff with vegetative growth (Muller-Starck & Seifert 2008; Ozolinčius et al. 2009).

Both drought and heat have been used to increase cone production in conifer seed orchards, but the timing of treatments is critical (Puritch 1972; Ross 1985; Schmidt 1993; Smith & Greenwood 1997). When applied during the period of strobilus initiation and differentiation, cultural treatments can influence the number of reproductive buds that develop. Once lateral shoot elongation has ceased in mid-summer, the fate of lateral buds has been determined and cannot be changed by subsequent treatments, such as those applied the following spring to influence subsequent strobilus development (Owens & Blake 1985).

Many areas in the Midwestern United States where Fraser fir is grown as a Christmas tree are much warmer and drier than the southern Appalachians. For example, the average summer temperature is 20.6°C in south central Michigan, compared with 16.1°C in Roan Mountain, TN (36.1942, -82.0683), a common seed source for Fraser fir used by Christmas tree growers. Moreover, annual rainfall is 81.7 cm in south central Michigan, and 130.9 cm in Roan Mountain (NOAA 2010). This climatic difference may explain the early and heavy cone production often apparent in Fraser fir trees in Midwestern plantations.

The objectives of this study were to evaluate the effects of drought and heat stress on cone production in Fraser fir trees grown in Michigan plantations. We hypothesized that irrigation would reduce cone formation by improving soil moisture availability and reducing tree moisture stress. We further hypothesized that mulch application would reduce cone development by moderating soil temperatures and reducing evaporation from the soil.

4.2. MATERIALS AND METHODS

4.2.1. Site descriptions

In 2013, we established irrigation studies at two Christmas tree farms, Getty Tree Farms located in Manton, Michigan (north-central), and Wahmhoff Farms Nursery in Gobles, Michigan (south-west) (Table 4.1). Each plantation was established in 2006 at a density of 2990 trees/ha. Plantations had been irrigated since planting by dripline, and were sheared each year to obtain the desired tree shape. Data collection commenced in May 2013 and continued until October 2015.

4.2.2. Reproductive phenology

In *Abies* spp., reproductive bud development is tied to shoot phenology. Bud initiation occurs when lateral shoot growth slows in early summer, and differentiation is complete by the time shoots are fully elongated (Owens & Blake 1985). We previously developed a model to estimate timing of reproductive bud initiation and differentiation based on growing degree days (GDD) and shoot phenology (Enviroweather 2016). Our model estimates that bud initiation may occur as early as 650 GDD with differentiation complete by 900 GDD (base 5°C, from 1 March). For this study, we used our phenology model to establish treatment timing and as a framework for interpreting results.

4.2.3. Irrigation and mulch treatments

At each farm, we selected 20 rows of 25 trees, and divided adjacent rows into paired plots to control for variability within the field. Border rows were used to reduce exposure effects. Irrigation was randomly assigned to one row in each paired plot, retaining the other row as an untreated control. Treated rows were irrigated using pressure-compensating dripline. At the Manton site, irrigation treatments began in late May, several weeks prior to strobilus initiation, and continued into August, several weeks after strobilus differentiation was complete. Irrigated trees received 13 mm of water every 4 d., regardless of weather. At the Gobles site, farm managers provided irrigation as needed to offset rainfall deficit (reference evapotranspiration – rainfall). At Gobles, we randomly selected one tree in each row for mulch treatment, with a second tree of comparable size and cone number serving as a paired control. 70% of mulched and control trees had produced cones in 2013, averaging 36 cones per tree. We installed mulch treatments on 6 June, six days before bud initiation, based on our model. We applied wood chip mulch 5 – 10 cm deep, in a ring extending 45 – 60 cm outward from the drip line of the tree. Mulch was applied only once in 2013, but was still present in subsequent years.

4.2.4. Environmental data collection

Precipitation data were collected from Next-Generation Radar (NEXRAD) stations operated by the National Oceanic and Atmospheric Administration (supplied in 5km resolution by Agrible, Inc., Champaign, IL). Reference evapotranspiration data were collected from Michigan State University Enviro-weather automated weather network stations located 16.9 km from the Gobles site and 1.4 km from the Manton site. Soil moisture was measured in three randomly selected paired plots in all treatments, using a volumetric soil moisture probe (10HS, Decagon Devices, Inc., Pullman, WA) installed at a depth of 30 cm, 20 cm out from the dripline, in line with the row. Soil temperature was measured in two paired plots in all treatments, using a temperature probe installed at a depth of 5 cm and centered between trees. Soil moisture and temperature measurements were taken every 60 s, and the average of the 60-s readings was recorded every hour throughout the growing season by a data logger (Em5b, Decagon Devices, Inc., Pullman, WA). We constructed the temperature probes using a 10 K thermistor (NTCLE413, Vishay Intertechnology, Malvern, PA) configured in a DC half-bridge with a 10 K reference resistor (MFP-25BRD52-10K, Yageo America, San Jose, CA). Probe accuracy was ± 1% (± 0.9 °C at 0 °C; ± 0.4 °C at 40 °C).

4.2.5. Coning and growth data collection

Each May, coning frequency (% of trees coning) and density (number of cones/tree) were measured for all trees. Once vegetative growth was complete (by late July), we measured terminal leader length for all trees. In addition, we measured shoot length, needle length, and bud density (buds/cm) for one representative, current-season lateral shoot in the upper few whorls on the south side of each tree.

4.2.6. Carbon isotope analysis

In October 2015, we collected needle samples for stable carbon isotope analysis in order to quantify the impact of irrigation and mulch on tree moisture stress. For each treatment, we collected five samples, each consisting of six shoots. Each shoot was collected from an unshaded branch in the upper three whorls on the south side of each of six randomly-selected trees within a treatment. Each shoot was separated into current-year and previous-year segments, rendering five samples for each treatment for 2014 and for 2015. Samples were dried at 70°C for three days, then needles were separated from stems, ground to 40 mesh (0.420 mm), and packed in tin capsules for shipment to the analytical laboratory. The ratio of ^{13}C to ^{12}C ($\delta^{13}C$) in needle samples was determined at the Center for Stable Isotope Biogeochemistry at the University of California-Berkeley using an IsoPrime 100 mass spectrometer (Isoprime Ltd., Stockport, UK). We used this ratio to calculate carbon isotope discrimination ($\Delta^{13}C$)—which serves as an integrated measure of moisture stress throughout the period of active growth—following the equations summarized in Cregg (2000). Needle samples were also pooled by treatment and sent to a commercial analytical laboratory (Waters Agricultural Laboratories, Inc., Camilla, GA) for nutrient analysis.

4.2.7. Experimental design and statistical analysis

Effects of treatments on growth were tested by analysis of variance based on a completely randomized design using PROC MIXED in SAS v9.4 (SAS Institute Inc., Cary, NC, USA). Means separation was accomplished using Tukey's honestly significant differences (HSD). The non-parametric Kruskal – Wallis test (PROC NPAR1WAY) was used to test differences in cone production because data failed to satisfy parametric assumptions even when data was transformed. The effects of treatments on soil temperature, soil moisture content, and foliar $\Delta^{13}C$ were tested using paired t-tests in PROC TTEST. All treatment effects were tested at $\alpha = 0.05$.

4.3. RESULTS

4.3.1. Manton

We analyzed each site independently since treatments were implemented differently between our two study locations. For the Manton site, we analyzed each year separately because of treatment x year interactions. Irrigation did not affect cone production in any year during this study, but it did increase growth in 2014 (Table 4.2). Cone production was highest in 2014, when density ranged from 0 – 370 cones/tree, compared with 0 – 220 in 2015. Coning frequency (trees with cones) did not vary by treatment or year, and averaged 52 ± 5.1%. Leader growth ranged from 6 cm – 80 cm in 2014 and 2015. In 2014, irrigation increased the length of terminal leaders, lateral shoots, and needles by 11%, 12%, and 7%, respectively. Bud density was unaffected by treatment in any year, and ranged from 0.18 – 1.20 buds/cm. Lateral shoot length and needle length increased each year from 2013 – 2015, while bud density decreased, regardless of treatment. Irrigation did not affect $\Delta^{13}C$, which averaged 20.87 ± 0.27‰ for irrigated trees and 21.26 ± 0.27‰ for control. The concentrations of all nutrients in needle samples were similar for treated and control trees and within the expected range for Fraser fir trees grown in Michigan plantations (Shelton 1997; Rothstein & Lisuzzo 2006).

The period of reproductive bud initiation and differentiation estimated by our phenology model was 24 June – 10 July in 2013, and 29 June – 19 July in 2014. We analyzed differences in soil moisture and temperature for those dates, and for 3 June – 28 July, the entire growing period covered by our sensors. Irrigation increased soil moisture each year and throughout the growing season, including during reproductive bud determination (Fig. 4.1). Daily means of volumetric soil moisture during the growing season ranged from a low of 11% in 2013 to a high of 28% in 2014. During bud determination, irrigation increased soil moisture by 7.0 ± 0.14 percentage points in 2013 and 1.1 ± 0.29 in 2014.

Soil temperatures varied between treatments and between years (Fig. 4.2). During the growing season, average daily soil temperatures ranged from 15.89 – 28.6°C in 2013, and from 16.0 – 24.3°C in 2014. Irrigation decreased mean daily soil temperatures by 1.7 ± 0.15°C in 2013, and by 1.98 ± 0.19°C in 2014. During bud determination, mean daily soil temperatures ranged from 20.1 – 25.6°C in 2013 and from 16.3 – 24.3°C in 2014. Respectively, irrigation decreased mean and maximum temperatures by 1.42 ± 0.18°C and 0.72 ± 0.22°C in 2013, and by 1.16 ± 0.10°C and 0.49 ± 0.15°C in 2014.

4.3.2. Gobles

The Gobles site received ample precipitation (Fig. 4.3), and irrigation was not applied during active growth including the critical period of reproductive bud determination in any year during this study. Therefore, only the effects of mulch treatments were analyzed, and were found to vary by year. Mulch applied in 2013 reduced 2014 mean cone density (average number of cones per tree) by 51% (Table 4.3). Cone density ranged from 0 –109 cones/tree for mulched trees, and from 0 – 209 for non-mulched trees. Mulch did not affect 2015 cone density, which ranged from 0 – 168 cones/tree. Coning frequency (% of trees producing cones) did not vary between treatments in any year, and ranged from an average of 78% in 2014 to 92% in 2015. Growth and development parameters varied considerably from tree to tree and from year to year, but did not differ between treatments in any year measured. In 2013, only needle length was measured. Needles were 22% shorter in 2013 than in subsequent years, and ranged from 10.0 – 19.8 mm in 2013 and from 11.2 – 26.1 mm in 2015. Leader length decreased by 17% from 2014 to 2015, and ranged from 29 – 79 cm in 2014 and from 25 – 88 cm in 2015. Bud density was similar in 2014 and 2015, and ranged from 0.23 – 1.00 buds/cm. Mulch did not affect $\Delta^{13}C$, which averaged 21.05 ± 0.08‰ for mulched trees and 21.14 ± 0.08‰ for control in 2014. Individual nutrient concentrations (or content) in needle samples were similar for mulched and control trees and within the expected range for Fraser fir trees grown in Michigan plantations (Shelton 1997; Rothstein & Lisuzzo 2006).

Our phenology model predicted reproductive bud initiation to begin on 12 June in 2013, and on 11 June in 2014, with differentiation complete by 26 June in both years. Therefore, we analyzed differences in soil moisture and temperature from 11 – 26 June and from 1 June – 31 July of each year, representing the period of bud determination and the general growing season, respectively.

The effects of mulch on soil temperature were consistent between years, with mulch decreasing mean and maximum soil temperature during the period of bud determination and throughout the growing season (Fig. 4.4). During the growing season, average daily soil temperatures ranged from 17.9 – 31.1°C, and mulch decreased soil temperatures by an average of 1.4°C in 2013 and 1.1°C in 2014. From 11 – 26 June, average daily soil temperatures ranged from 19.5 – 28.0°C in 2013, and from 18.0 – 25.8°C in 2014. The highest soil temperature recorded during bud determination was 36.8°C for control in 2013—10 degrees warmer than soil under mulched trees. During this same period, mulch lowered the average maximum daily soil temperatures 7.4 ± 0.72°C in 2013 and 4.8 ± 0.69°C in 2014.

Mulch increased soil moisture throughout each growing season, but not necessarily during bud determination (Fig. 4.5). From 1 June – 31 July, volumetric soil moisture ranged from 21 – 30%, and mulch increased soil moisture by 1.9% in 2013 and by 3.6% in 2014. During bud determination, mulch increased average soil moisture content by 0.16 ± 0.062 percentage points in 2013, but did not affect soil moisture in 2014.

4.4. DISCUSSION

Irrigation (Manton site) and mulch (Gobles site) increased soil moisture content and reduced soil temperatures. However, cone production was only affected at Gobles, and only in 2014. This disparity is likely resolved by examining differences in weather between sites and years. At Gobles, soil temperatures during lateral bud determination were much more heavily influenced by mulch in 2013 than 2014. Although the actual temperatures of the soil under mulch were comparable between years (mean 21°C, max 23°C), the difference in average daily temperature between mulch and control was 1.0°C higher in 2013 than in 2014, and the difference in maximum soil temperature was 2.6°C higher. This suggests that soil temperatures did not rise enough in 2014 to distinguish between treatments. Although rainfall was equal to or greater than reference evapotranspiration in both years (Fig. 4.3), the pattern of rain events varied by year. In 2013, rain occurred in roughly alternate weeks during June and July, compared with much more consistent rainfall in 2014. In fast-draining loamy sand, gaps in rainfall in 2013 may have allowed mild, transient moisture stress conditions to develop.

Mulching would have reduced water loss through evaporation, which may be reflected in the modestly higher soil moisture content under mulched trees in 2013.

Manton is further north and therefore cooler than Gobles. During differentiation, the maximum temperature under any treatment at Manton was 25.6°C, which is 0.4°C lower than the maximum temperature under mulch at Gobles. This suggests that, like in 2014 at Gobles, the temperatures were not high enough to discriminate between treatments. Drought conditions existed at Manton throughout much of the 2013 growing season. The rainfall deficit (reference evapotranspiration – rainfall) from 1 June – 31 July was 138 mm in 2013 and 83 mm in 2014. Soil moisture was 47% higher in irrigated plots during differentiation in 2013, but there was no effect on cone production. This suggests that drought stress alone is not sufficient to explain heavy cone production in Fraser fir. Similarly, studies in Norway spruce suggest that high temperatures during cone initiation are a stronger determinant of cone production than precipitation, though precipitation may remain a significant factor (Solberg 2004).

It is also possible that the treatment effects noted at Gobles were influenced by the unusual weather of 2012. In 2012, much of the central United States experienced extreme heat and drought, which extended to the Gobles site, but not to Manton. Although heat and drought often trigger high cone production (Owens & Blake 1985), cone production in 2013 was not unusually high at Gobles (36 cones/tree). However, needles were unusually short in 2013 despite the cooler temperatures and consistent precipitation favorable to growth, likely because of carryover effects from the drought of 2012. Also, extreme or prolonged drought may decrease cone production (Ozolinčius et al. 2009), particularly when it extends beyond the period of reproductive bud differentiation (Ross 1985), possibly because of death of the newly differentiated buds (Ebell 1971). It may be that the trees lacked the necessary resources to support a heavy cone crop into 2013 following the drought of 2012, but retained internal stress signals that predisposed them to higher strobilus production, making them more sensitive to treatment differences (environmental signals) in 2013 (see Koenig & Knops 2000; Sala et al. 2012; Roland et al. 2014).

In conclusion, our findings suggest that high soil temperatures are associated with cone formation in Fraser fir, and application of mulch, irrigation, or other treatments that reduce root-zone temperatures may reduce cone formation in highly inductive (i.e., hot) locations or years. It is unclear whether cone formation may be further suppressed by treatments that lower soil temperatures below those reached in this study, or whether other endogenous or environmental factors govern reproductive development at those lower temperatures. Although soil moisture stress may interact with heat stress or other environmental signals to enhance cone formation, drought stress alone was not sufficient to stimulate cone production.

APPENDIX

Table 4.1. Site descriptions of study locations in Michigan, 2013.

	Coordinates (decimal degrees)		Soil		Ave. temperature[b] (°C)		Total precipitation[b] (cm)		Ave. tree height (m)
City	Latitude	Longitude	Type[a]	pH	Annual	June	Annual	June	
Gobles	42.3751	-85.8707	Spinks loamy sand	6.0	10.9	20.1	90.8	9.1	1.60 ± 0.02
Manton	44.4026	-85.2775	Rubicon sand	5.3	9.0	18.4	87.3	9.0	1.65 ± 0.01

a. Web soil survey (USDA NRCS).

b. 1981 – 2010 U.S. Climate Normals (NOAA 2010).

Table 4.2. Mean cone density and growth of Fraser fir trees in response to irrigation. Manton, Michigan.

	Coning frequency[a] (%)		Cone density[b] (cones/tree)		Leader length (cm)		Lateral length (cm)		Needle length (mm)		Bud density (buds/cm)	
Treatment	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Irrigated	51.0	57.0	26.4	22.4	48.9*	48.1	20.9*	27.9	16.9*	19.0	0.52	0.43
Control	48.0	53.3	28.4	16.7	43.9	50.2	18.7	27.0	15.8	18.7	0.54	0.45

a. Percent of trees that produced cones.

b. Means did not differ from control (Kruskal – Wallis test).

* indicates significant difference at α = 0.05 (ANOVA).

Table 4.3. Mean cone density and growth of Fraser fir trees in response to mulch. Gobles, Michigan.

	Coning frequency[a] (%)		Cone density (cones/tree)		Leader length (cm)		Lateral length (cm)		Needle length (mm)		Bud density (buds/cm)	
Treatment	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Mulch	80.0	100.0	27.9*	62.1	59.3	50.1	21.5	17.2	16.6	16.7	0.48	0.43
Control	75.0	84.2	57.4	54.8	59.6	49.0	23.0	18.7	17.2	17.3	0.51	0.49

a. Percent of trees that produced cones.

* indicates significant difference in mean cone density at α = 0.05 (Kruskal – Wallis test). Other means did not differ from control (ANOVA).

Figure 4.1. Soil moisture under irrigated and non-irrigated Fraser fir trees, measured 30 cm below the soil surface. Manton, MI.

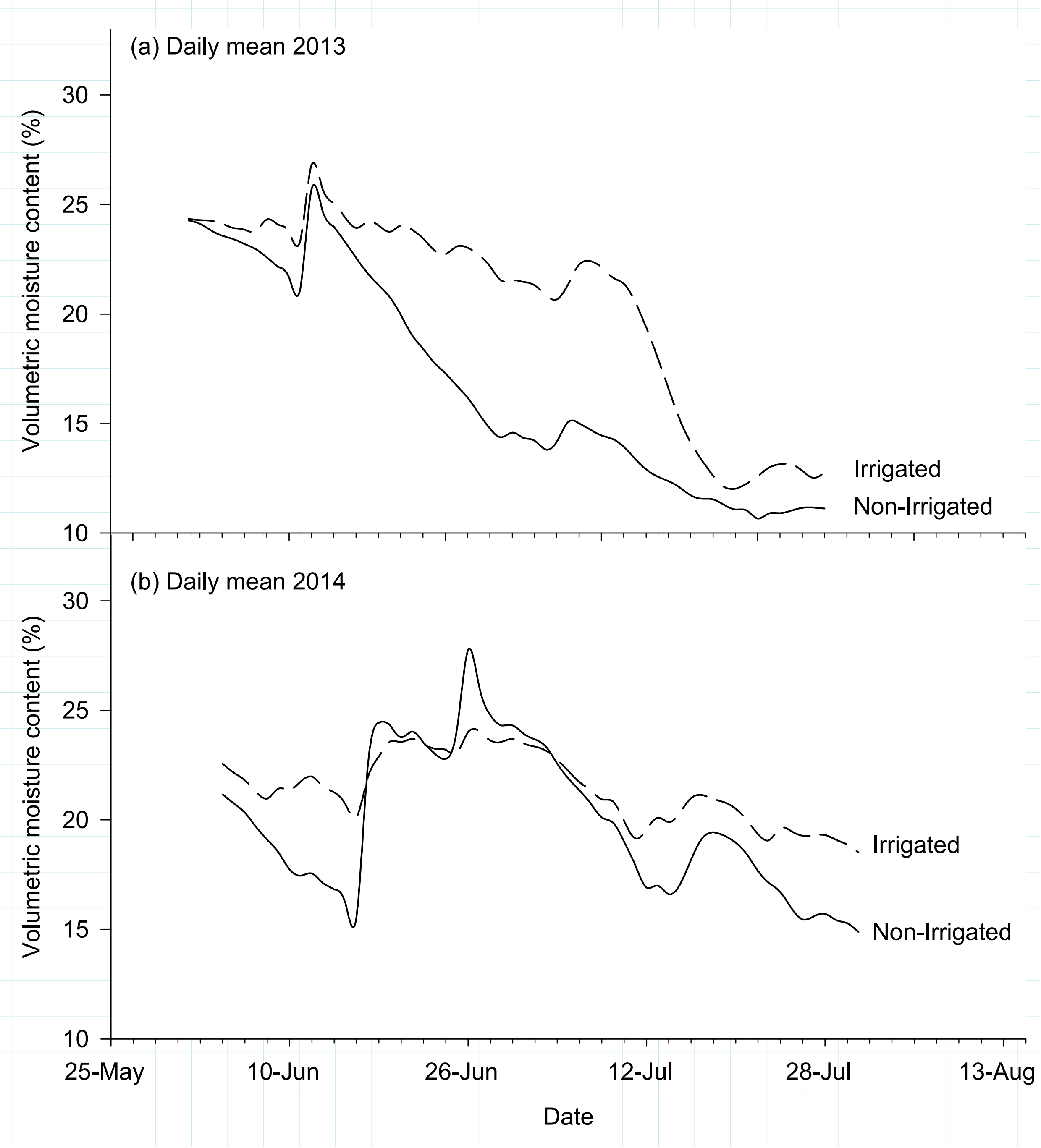


Figure 4.2. Average daily soil temperatures under irrigated and non-irrigated Fraser fir trees, measured 5 cm below the soil surface, between trees within rows. Arrows indicate period of reproductive bud differentiation based on our phenology model for Fraser fir. Manton, MI.

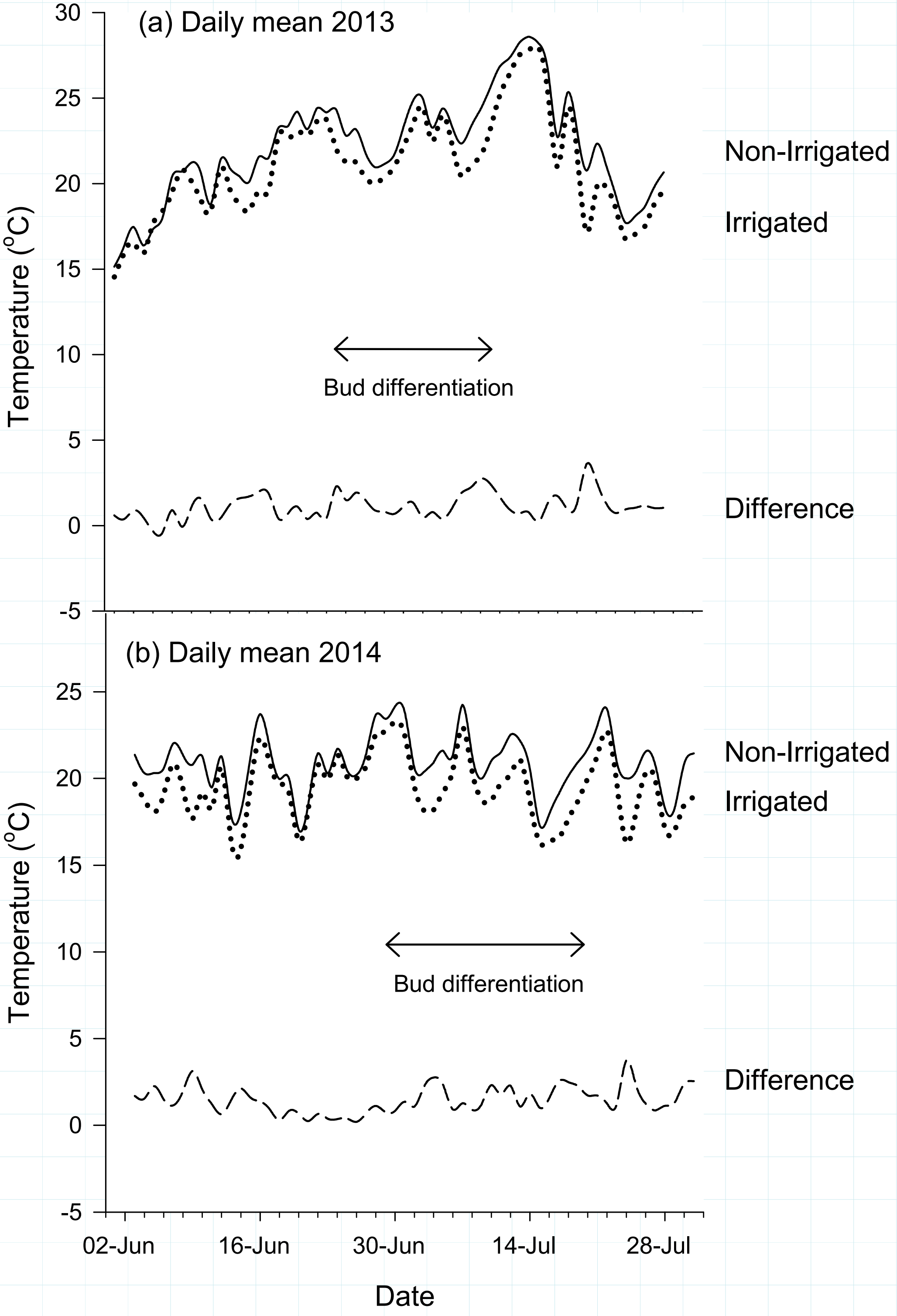


Figure 4.3. Weekly rainfall and reference evapotranspiration (RET), Gobles, MI. Rainfall data is compiled from Next-Generation Radar (NEXRAD) stations. RET data is from Michigan State University Enviro-weather automated weather network station located 17 km from the site. Arrows indicate period of active shoot growth for Fraser fir trees.

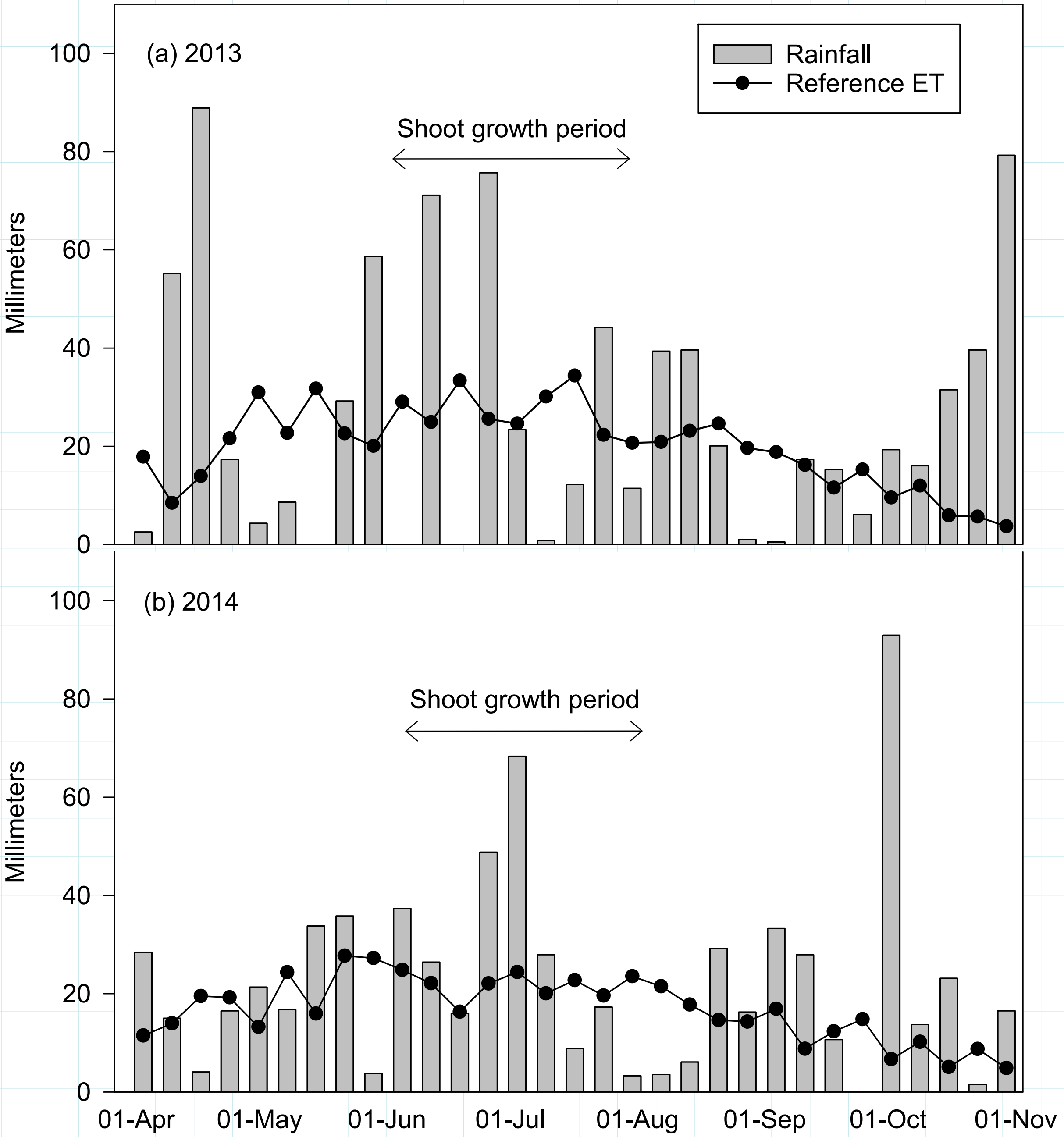


Figure 4.4. Average maximum daily soil temperatures under mulched and non-mulched Fraser fir trees, measured 5 cm below the soil surface, between trees within rows. Arrows indicate period of reproductive bud differentiation based on our phenology model for Fraser fir. Gobles, MI.

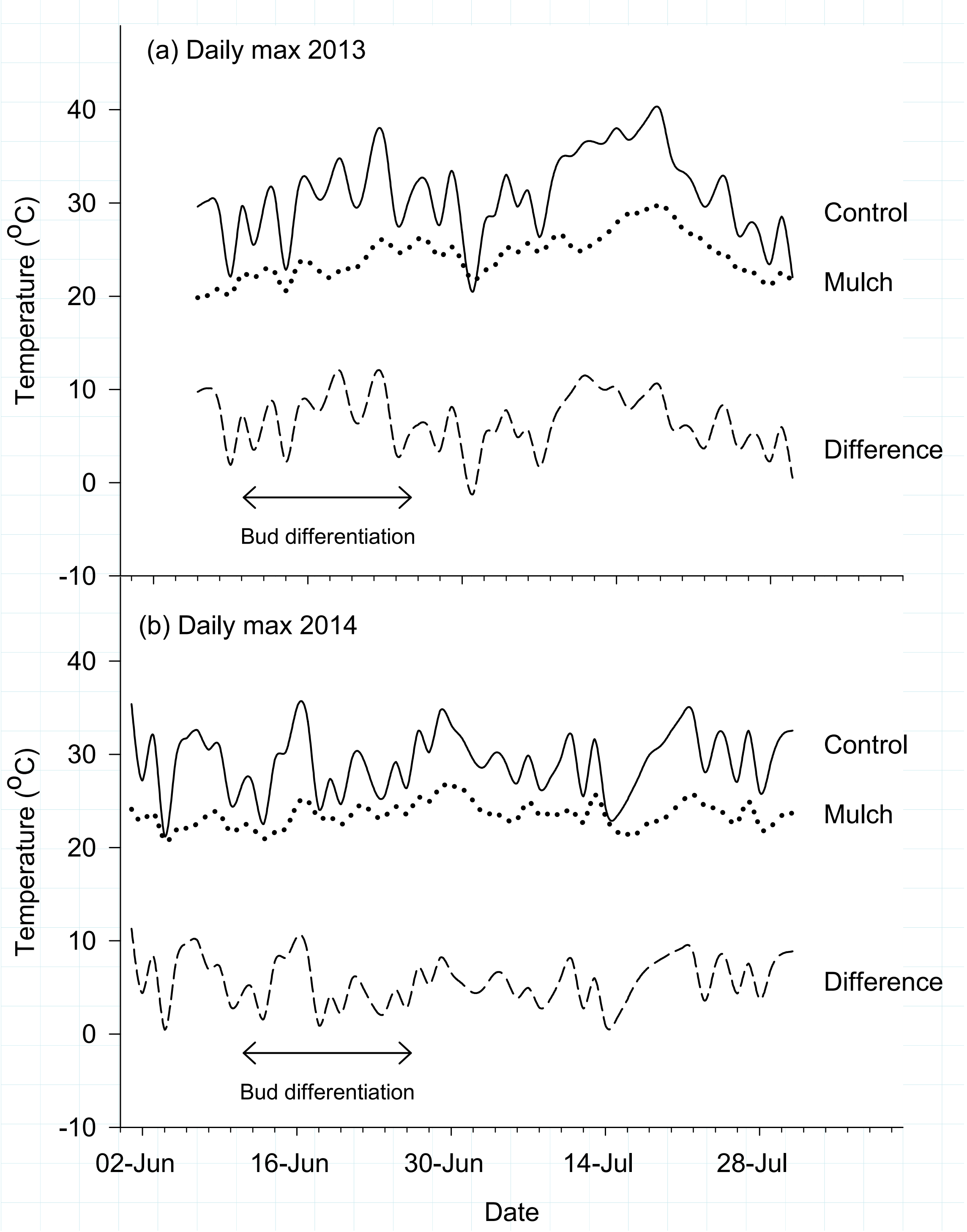
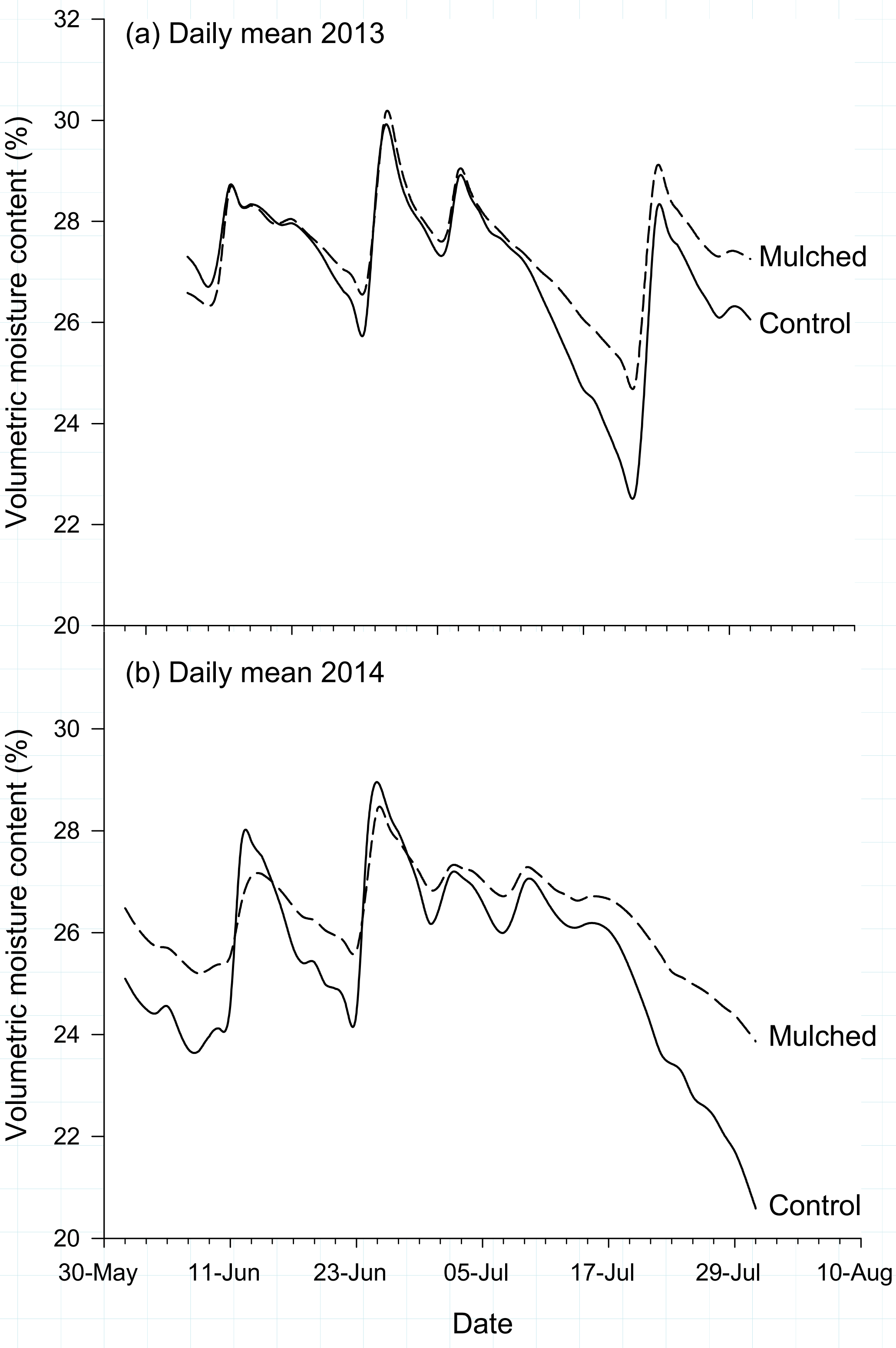


Figure 4.5. Soil moisture under mulched and non-mulched Fraser fir trees, measured 30 cm below the soil surface. Gobles, MI.



CHAPTER FIVE

DOES SHOOT TEMPERATURE REGULATE CONE FORMATION IN FRASER FIR (*ABIES FRASERI*)?

ABSTRACT

DOES SHOOT TEMPERATURE REGULATE CONE FORMATION IN FRASER FIR (*ABIES FRASERI*)?

The ability to influence strobilus (cone) formation in *Abies* is important both for Christmas tree growers who want to reduce costs associated with undesired cone production and for seed orchard managers who require consistent seed production. Heat and drought are important environmental regulators of reproduction in mast-seeding species, and have been used to increase cone production in conifers, including *Abies amabilis*. In Fraser fir (*Abies fraseri*), we tested the effects of temperature on cone formation using overhead misting for evaporative cooling and polyethylene tenting for solar heating throughout the period of active growth, including the window of reproductive bud initiation and differentiation. Misting reduced average daily temperatures of lateral shoots where cones develop by 2.4°C and daily maximum temperatures by 5.0°C, while also increasing soil moisture by 20%. Tenting increased average daily shoot temperatures by 1.4°C and maximum temperatures by 3.8°C, while increasing soil moisture by 12%. Neither misting nor tenting affected cone production, suggesting that cone formation in Fraser fir is regulated by factors other than temperature at the developing bud.

5.1. INTRODUCTION

Many conifers species are mast-seeding, with seed production highly variable across years, yet synchronized across a population (Kelly 1994). The mechanisms governing mast-seeding are not well understood, but weather conditions at the time of cone initiation and differentiation are important drivers (Roland et al. 2014). In particular, high temperatures and dry conditions during the summer increase subsequent cone production in many conifers, including pine, spruce, and fir (Owens & Blake 1985; Solberg 2004; Messaoud et al. 2007). Tenting (covering a tree with polyethylene to trap solar heat) has been used in seed orchards as one of many cultural and chemical treatments designed to increase cone initiation, particularly under non-inductive conditions (e.g., cool, wet summers) (Owens & Blake 1985). Although cultural treatments are generally most effective in combination with GAs, tenting alone may increase cone production in some conifers, such as Sitka spruce (Tompsett 1977). To our knowledge the effects of heat treatment have been reported for only one *Abies* species: Tenting Pacific silver fir (*Abies amabilis* [Douglas ex Loudon] Douglas ex Forbes) significantly increased the effectiveness of other treatments ($GA_{4/7}$ + fertilizer + girdling), although tenting alone was not tested (Owens et al. 2001).

While seed orchard managers want to increase cone production, Christmas tree growers in the United States want to reduce cone production. Fraser fir (*Abies fraseri* [Pursh] Poir.) is an important Christmas tree species in the Midwest and in the southern Appalachians, where annual sales exceed 100 million USD (Pettersson et al. 2015). Grown outside of its native environment, such as in plantations in Midwestern states, young Fraser fir trees produce abundant, intermittent cone crops (Cregg et al. 2003). Developing cones consume photosynthates at the expense of vegetative growth, and leave behind unsightly stalks and resinous scales upon disintegration in early fall (Crain et al. 2012). To produce salable trees growers must remove cones by hand, which is labor-intensive (Cregg et al. 2003). Even in plantations in the southern Appalachians within the native range of Fraser fir, heavy cone production is a growing problem on larger trees. In that market, supply of Fraser fir trees has outpaced demand for several years, allowing many trees to grow large enough to begin producing cones, particularly following periods of heat and drought. In both Michigan and North Carolina (Owen 2015), some producers report cone removal to be their most expensive cultural management practice, when considered on a per-tree basis.

Since warming trees by tenting increases cone production in Pacific silver fir (Owens et al. 2001), it seems likely that cooling of the tree crown might decrease cone production in Fraser fir. Evaporative cooling by overhead misting has been used effectively in conifer seed orchards to delay bud break for frost protection and reduction of outside-pollen contamination (Fashler & El-Kassaby 1987). Mist-cooling has also been used for frost protection in tree fruits, and may delay bloom by 7 – 10 days (Rijal et al. 2014). This delay is highly localized, with branches that evade mist—such as those growing above the reach of the sprayers—breaking bud in synchrony with control trees. This indicates that timing of bud break is regulated by temperature at (or near) the developing bud, consistent with findings that meristem temperature, rather than air temperature, regulates phenology in plants (Savvides et al. 2013).

The purpose of the current study was to test whether the temperature at the lateral bud locally regulates cone initiation and differentiation in Fraser fir. Two methods were used, one that has been used in seed orchards to increase cone production, and another that could be used by Christmas tree growers to reduce cone production. Tenting was used to increase temperature in the upper crown by solar heating, and a misting system was used to decrease temperature in the upper crown by evaporative cooling.

5.2. MATERIALS AND METHODS

In 2014, the study was established at Michigan State University's Southwest Michigan Research and Extension Center (Benton Harbor, Michigan). The site (42°05'17.2"N 86°21'28.1"W) had been planted for a study in 2006 in four 8-row blocks of alternating sets of spruce and fir trees on 2.1 m x 2.1 m spacing (Cregg et al. 2009). In mid-May 2014, Fraser fir trees were scored for current-year and prior-year seed cone production, and assigned a value of 0 for no cones, 1 for fewer than 20 cones, and 2 for 20 or more cones. Prior-year score was based on the number of cone stalks remaining from previous years. Since trees with high reproductive output were desired for the study, trees with an average current- and prior-year score of one or below were excluded. By late May, all cones had emerged and were counted on each tree and 54 trees were selected for study. The mean height (± SE) of selected trees was 2.43 (± 0.042) m. Trees were randomly assigned to one of three treatments: mist-cooling, tenting, and control. Twenty-one trees were selected for mist-cooling, 12 for tenting, and 21 were left untreated as controls. Fewer trees were selected for tenting because construction of additional tents would have exceeded available labor constraints. At least two buffer trees separated the trees that were misted from trees that were tented or control to prevent overspray by the misting system.

5.2.1. Mist-cooling treatment

Water was provided from an onsite well, run through a sediment filter, and regulated to 210 – 240 kPa before being delivered to trees using 18-mm polyethylene tubing and micro-sprayers (35 l/h nozzle, flat static spreader; NaanDanJain Irrigation Ltd., Israel). A micro-sprayer was attached a few cm below the top buds on the main stem of each treated tree, and connected to the leader using zip-ties or grafting bands painted with castor oil for UV resistance (Fig. 5.1). Polyethylene tubing (4-mm) connected the sprayer to the 18-mm polyethylene supply lines. Misting was controlled by an evaporative cooling application running on a datalogger (CR1000, Campbell Scientific, Logan, UT, USA) as part of a system developed in Michigan for delay of bud break in cherry and apple (Rijal et al. 2014). The datalogger activated the system by powering a solenoid on the main water supply line. Misting was based on evaporation rates calculated as a function of ambient air temperature and relative humidity (RH) measured at 1-min intervals at upper-canopy height using a combination temperature + RH sensor (HMP60, Vaisala Oyj, Helsinki, Finland). This sensor was housed in a multi-plate radiation shield (R. M.

Young Co., Traverse City, MI) on a weather station at the edge of the field. The misting system remained active while temperatures were above 15°C and RH was less than or equal to 90%. Misting was activated for 105 s, followed by a delay that varied from 750 s at 15 – 20°C and 75 – 90% RH to 210 s at > 24°C and 0 – 50% RH. The end result was that mist was reapplied just frequently enough to maintain some moisture on needle surfaces. Misting commenced on May 29 for 16 trees. Because of labor and time constraints, misting commenced on June 6 for the remaining 5 trees. Misting was discontinued on Aug. 15.

5.2.2. Tent-heating treatment

Four-sided, trapezoidal frames were constructed from 38 mm x 38 mm pine boards, overlaid with 0.15 mm polyethylene film, and placed over trees selected for tent-heating treatment (Fig. 5.2). The polyethylene film enclosed only the upper third to half of each tree canopy, leaving the lower canopy uncovered and the top of the tree open to prevent excessive heat build-up. Tents were installed on June 6 and removed on August 15.

5.2.3. Data collection

Shoot temperature (as a proxy for bud temperature) was measured in three randomly selected mist-cooled trees, and in two tented and control trees using thermistor temperature probes installed June 28. The temperature probe was built using a 10 K thermistor (NTCLE413, Vishay Intertechnology, Malvern, PA, USA) in a DC half-bridge configuration with a 10 K reference resistor (MFP-25BRD52-10K, Yageo America, San Jose, CA, USA). The thermistor tip was left exposed, and the remaining circuitry was encased in high adhesive flow heat shrink tubing (NSPA-HST540C-48, National Standard Parts Associates, Inc., Pensacola, FL, USA). Temperature was calculated by fitting a polynomial to the calibration curve as $T = -(y - 2200) / 27.5$, where T is temperature in degrees C and y is the output from the probe measured in mV.

Thermistor probe accuracy was ± 1%, yielding a temperature accuracy range from ± 0.9 °C at 0 °C to ± 0.4 °C at 40 °C. Temperature probes were embedded (1 per tree) into 1.5 x 2 mm holes drilled into 1-yr-old, lateral branches just below current-season growth, in the upper few whorls on the south side of the tree, but shaded from the sun. Soil moisture was also measured under these same trees. On July 2, volumetric moisture probes (10HS, Decagon Devices, Inc., Pullman, WA, USA) were installed at a depth of 30 cm just outside the tree dripline. Soil moisture and stem temperature readings were taken every 60 s, and the mean was recorded every hour throughout the remainder of the treatment period by a data logger (Em5b, Decagon Devices, Inc., Pullman, WA, USA).

Six trees in each treatment were randomly selected to track growth. For each tree, the length of the terminal leader and one lateral shoot in the upper third of the crown on the south side of the tree were measured periodically from late June to early August. At the end of the growing season, leader length, crown radius, lateral shoot length, and bud density (buds/cm) were measured for all trees. In early October, we collected soil samples randomly from 10 locations within each treatment. The soil cores were extracted from the surface of the mineral soil to a depth of 20 cm using a 1.3 cm soil recovery probe. Soil cores were separated into five replicates per treatment and analyzed for pH and EC.

We collected needle samples for stable carbon isotope analysis to determine the effects of misting and tenting on tree moisture and heat stress. In October 2015, we collected one lateral shoot that included needles from 2014 and 2015 from an unshaded upper branch on the south side of 12 – 15 randomly selected trees within each treatment. We divided the shoots into five replicate samples per treatment per year, and dried them for three days at 70°C. Needle tissue was separated from stems, ground to 40 mesh (0.420 mm), and packed in tin capsules. The

Center for Stable Isotope Biogeochemistry at the University of California-Berkeley used an IsoPrime 100 mass spectrometer (Isoprime Ltd., Stockport, UK) to determine the ratio of ^{13}C to ^{12}C ($\delta^{13}C$) in needle samples. From this ratio, we calculated carbon isotope discrimination ($\Delta^{13}C$) —which serves as an integrated measure of moisture stress during growth—following the equations summarized in Cregg (2000). Needles samples were also pooled by treatment and sent to a commercial analytical laboratory (Waters Agricultural Laboratories, Inc., Camilla, GA) for nutrient analysis. Because needles from misted trees were coated in a flakey residue, care was taken to remove the residue, and an additional sample was prepared (washed in DI water), to compare results. In May 2015, coning frequency (% of trees coning) and density (number of cones/tree) were recorded for all trees.

5.2.4. Data analysis

Effects of treatments on growth were tested by analysis of variance based on a completely randomized design using PROC MIXED in SAS v9.4 (SAS Institute, Chicago, IL, USA). Means separation was accomplished using Tukey's honestly significant differences (HSD). The non-parametric Kruskal – Wallis test (PROC NPAR1WAY) was used to test differences in cone production because transformation failed to satisfy parametric assumptions. The effects of treatments on soil temperature, moisture content, and $\Delta^{13}C$ were tested using a two-tailed Dunnett's test. All differences were tested at $\alpha = 0.05$.

5.3. RESULTS

5.3.1. Cone production

All trees in the trial produced cones in 2015, with the exception of a single control tree. Mean cone density (average number of cones/tree) did not vary among treatments. Comparison of cone production against a pre-treatment baseline for each tree showed no treatment effect (Table 5.1).

5.3.2. Shoot temperature and soil moisture

Evaporative cooling reduced daily mean temperature of misted shoots by 2.4°C (max 3.6°C, min 0.1°C) (Fig. 5.3) and midday (maximum) temperature by an average of 5.0°C (max 8.4°C, min -1.7°C) (Fig. 5.4) compared to shoots on control trees during the period covered by environmental sensor data. Solar heating of tented trees increased daily mean temperature by 1.4°C (max 2.6°C, min -0.1°C) and midday temperature by an average of 3.8°C (maximum 8.2°C, minimum -2.4°C) compared to control trees. Night (minimum) temperatures were similar in all treatments. All treatments affected the shape of the temperature response curve (Fig. 5.5). Temperatures of tented trees increased much more rapidly, and earlier in the day. Temperatures increased much more slowly in misted trees, yielding a much flatter response curve, even during cool, cloudy weather. Volumetric soil moisture content was higher under both misted trees (mean 0.30, max 0.31, min 0.28) and tented trees (mean 0.28, max 0.31, min 0.18) than control (mean 0.25, max 0.28, min 0.17). In general, moisture content in the soil under tented trees remained several percentage points higher than under control trees, but the shape of the response curve over time was similar (Fig. 5.6), with moisture content trending down through the summer. Moisture content in soil under misted trees was much higher than under tented or control trees, and remained near field capacity throughout the summer.

5.3.3. Vegetative growth

Leader growth was similar in all treatments, and was largely complete by mid-July, but slow growth continued until late July. Growth of lateral shoots varied among treatments. By July 10, lateral growth had ceased in tented trees and had reached > 95% completion in all other trees. By July 17, lateral growth was complete in control trees, but very slow growth (< 1 cm) continued into late July in misted trees. Growth rates were highly variable within and among treatments, and there were no differences in any of the final growth parameters (Table 5.2).

5.3.4. Foliar and soil analyses

$\Delta^{13}C$ was the same in needles of misted trees (21.45 ± 0.14‰), control trees (21.47 ± 0.14‰), and tented trees (21.06 ± 0.12‰), indicating that treatments had no effect on stomatal conductance. Nutrient concentrations and content in needle samples were similar for tented and control trees and within the expected range for Fraser fir trees grown in Michigan plantations (Shelton 1997; Rothstein & Lisuzzo 2006). However, Fe, Ca, and Mg were much higher in misted trees (139.7 ppm, 1.71%, and 0.12%, respectively) than control (55.7 ppm, 0.47%, and 0.09%), in both washed and unwashed needles. Misting over two seasons increased soil pH from 5.02 ± 0.09 to 5.6 ± 0.09, compared to control. Tenting did not affect pH (4.86 ± 0.09). EC did not vary among treatments, and averaged 0.06 ± 0.01 μS/cm.

5.3.5. Tree injury

A few damaged or dead shoots were observed in the upper crown of tented trees during the treatment period; insect damage was apparent in some cases. Damage was more severe in misted trees, four of which died in the fall following treatment (Fig. 5.7). No other tree mortality was evident in the field. Needle surfaces of misted trees were covered by a white, flaky residue. In trees that died, misted needles turned chlorotic, then reddish-brown, generally progressing from the interior of the tree outward over a period of several weeks, and buds on the affected branches died. Non-misted branches lower in the tree remained green for at least a few months, but it seemed unlikely that the tree would recover. Plant materials in various stages of decline—

including one whole tree—were examined by MSU Diagnostic Services (East Lansing, MI). No association was found with pathogens that could explain the observed decline.

5.4. DISCUSSION

5.4.1. Shoot temperature and soil moisture

Although environmental sensors were not installed until the end of bud differentiation, they provide insight into the effectiveness of the treatments. Shoot temperature was consistently higher during the day for tented trees, and lower for misted trees, as intended. However, soil moisture content also changed in response to treatments, and it was not possible for us to separate the effects of changes in soil moisture from the effects of changes in temperature. Soil moisture remained near field capacity under misted trees, suggesting that efficiency of the water delivery system may be improved. However, this also indicates that no additional irrigation is required when this misting system is in use, and no increase in soil-pathogen activity (e.g., *Phytophthora* root rot) was observed. Soil moisture remained somewhat higher under tented trees than control trees, despite the increase in temperatures within the tents. Condensation was consistently observed on the inside of the polyethylene tent material, and dripping of this condensation may explain the higher soil moisture levels.

5.4.2. Growth

Growth of lateral shoots was affected by treatments, with incremental growth continuing in misted trees until around the time that leader growth was complete, a few weeks after growth had ceased in other treatments. This persistent growth was likely the result of lower temperatures, since phenology is regulated by thermal time (Trudgill et al. 2005; Cleland et al. 2007). However, cessation of leader growth was similar in all treatments, suggesting that growth cessation and entrance into dormancy are more likely under the control of photoperiod than temperature, as in other species (Ekberg et al. 1979; Rossi et al. 2006).

5.4.3. Tree Stress

^{12}C is preferentially incorporated into plant tissue at the expense of the heavier ^{13}C, which is discriminated against during photosynthesis. Stress factors that reduce stomatal conductance, such as drought and heat, decrease the ratio of ^{12}C to ^{13}C in plant tissue (Farquhar 1989). Stable carbon isotope analysis uses that ratio to provide a single measure of discrimination, $\Delta^{13}C$, which integrates information on cumulative tree stress throughout the growing season. In this study, neither misting nor tenting affected $\Delta^{13}C$, which remained at levels associated with lower stress in other studies (e.g., Cregg 2005; Taylor et al. 2013). This lack of treatment effect is surprising, and indicates that temperatures and soil moisture were not limiting to photosynthesis in 2014, even under tented conditions.

5.4.4. Cone production

It was surprising that treatments had no effect on cone production, particularly given the dramatic enhancement in cone production under nearly identical conditions in tented Pacific silver fir (Owens et al. 2001). However, cultural treatments are rarely effective for cone enhancement unless combined with GA, and tenting alone was not tested in Pacific silver fir (Owens et al. 2001). Timing is also critical, and treatments may have been applied too early. For example, in spruce, heat does not promote cone production when initiated during early, rapid shoot elongation, but is promotive when applied toward the end of lateral shoot expansion, during reproductive bud initiation (Ross 1985). This promotive effect may be due to heat-induced changes in GA metabolism (Chałupka et al. 1982). In the Pinaceae, less-polar GAs are involved in reproductive determination (Owens 1995). In Norway spruce, these less-polar GAs increase after one day of tenting, and remain elevated for 2 to 3 weeks (Chałupka et al. 1982). In spruce and fir, reproductive bud initiation occurs during late shoot elongation, followed by anatomical differentiation over the next 2 – 3 weeks (Owens & Blake 1985). Therefore, to maintain elevated levels of promotive GAs throughout bud differentiation, heat treatments should be timed to coincide as closely as possible with bud initiation during late shoot elongation. When applied early in the season, the elevated levels of endogenous GAs may have reverted to baseline prior to reproductive differentiation. In the case of Pacific silver fir (Owens et al. 2001), tenting was applied at vegetative bud break at a high elevation site with much cooler temperatures than in this study. At our site, reproductive bud initiation likely occurred in mid-June in control trees, earlier in tented trees, and later in misted trees, based on shoot phenology. Treatments were set in place two to three weeks prior to strobilus initiation. If tenting increased the levels of promotive GAs, they would likely have returned to baseline prior to reproductive initiation.

5.4.5. Tree injury

Although the problem was not widespread, some shoots were damaged in the upper crown of tented trees. Most damage appeared to be the direct result of high temperatures within the tent, although tents did provide cover for insects, and high temperatures and humidity that could promote disease. Damage to shoots may account for a portion of the reduction in cone production observed in tented trees.

Damage to misted trees was more severe, resulting in the death of 4 of the 21 trees. In the early stages of tree decline, needle and bud necrosis was limited to misted branches, indicating that the eventual mortality was the direct or indirect result of misting. Although needles turned reddish-brown (Fig. 5.7), the temporal pattern was not consistent with

Phytophthora root rot, and a thorough examination of plant material indicated that pathogens were not involved. The water used for misting was very hard, 193 mg/l as $CaCO_3$. Fe, Ca, and Mg concentration were much higher in needles of misted trees, reflecting the quality of the water used for misting. The white, flakey material observed on misted needles was probably a buildup of Ca and Mg salts deposited throughout the summer as water evaporated from the needles. Symptoms were similar to those observed in conifers exposed to deicing salts from nearby roadways (Barrick 1979). Mortality was likely a direct result of salt toxicity.

In conclusion, misting increased soil moisture content and reduced lateral shoot temperatures in Fraser fir. However, it had no effect on cone production, and cannot be recommended as a cultural practice to reduce heavy cone production in plantation-grown Christmas trees. Tenting of trees increased both lateral shoot temperature and soil moisture content, but likewise had no effect on cone production. It is possible that combining misting with GA inhibitors may result in reduced cone production, and likely that combining tenting with GA will increase cone production, as in Pacific silver fir (Owens et al. 2001). In summary, neither mist-cooling nor warming by tenting affected cone formation, suggesting that cone formation in Fraser fir is regulated by factors other than temperature.

APPENDIX

Table 5.1. Treatment effects on cone production in *A. Fraseri*, Benton Harbor, MI, 2014.

Treatment	n	Coning frequency (%) [a]	Cone density [b]	Cone difference [c]
Control	25	96	81.2	61.7
Spray	17	100	80.8	63.1
Tent	11	100	73.8	55.3

a. Percent of trees producing cones; *b*. average number of cones per tree; *c*. difference between cones produced in 2015 and 2014 (pre-treatment baseline). Means did not differ among treatments at α = 0.05 (Kruskal – Wallis test).

Table 5.2. *A. Fraseri* growth (mean ± SE) by treatment, Benton Harbor, MI, 2014.

Treatment	n	Leader length (cm)	Lateral length (cm)	Crown radius (cm)	Bud density (no./cm)
Control	25	48.0 ± 2.7	20.9 ± 1.2	86.4 ± 2.0	12.2 ± 0.57
Spray	21	42.0 ± 3.0	23.4 ± 1.3	87.6 ± 2.2	10.9 ± 0.62
Tent	12	42.3 ± 4.0	21.2 ± 1.7	83.3 ± 2.9	11.1 ± 0.82

Means did not differ significantly among treatments at α = 0.05 (ANOVA).

Figure 5.1. Micro-sprayer delivering mist for evaporative cooling of upper tree canopy.

Figure 5.2. Polyethylene tent used for solar heating of upper tree canopy.

Figure 5.3. Average daily temperatures of shoots of Fraser fir trees that were tented, misted, or control. Bottom set of lines represents differences between temperatures of tented or misted shoots and control.

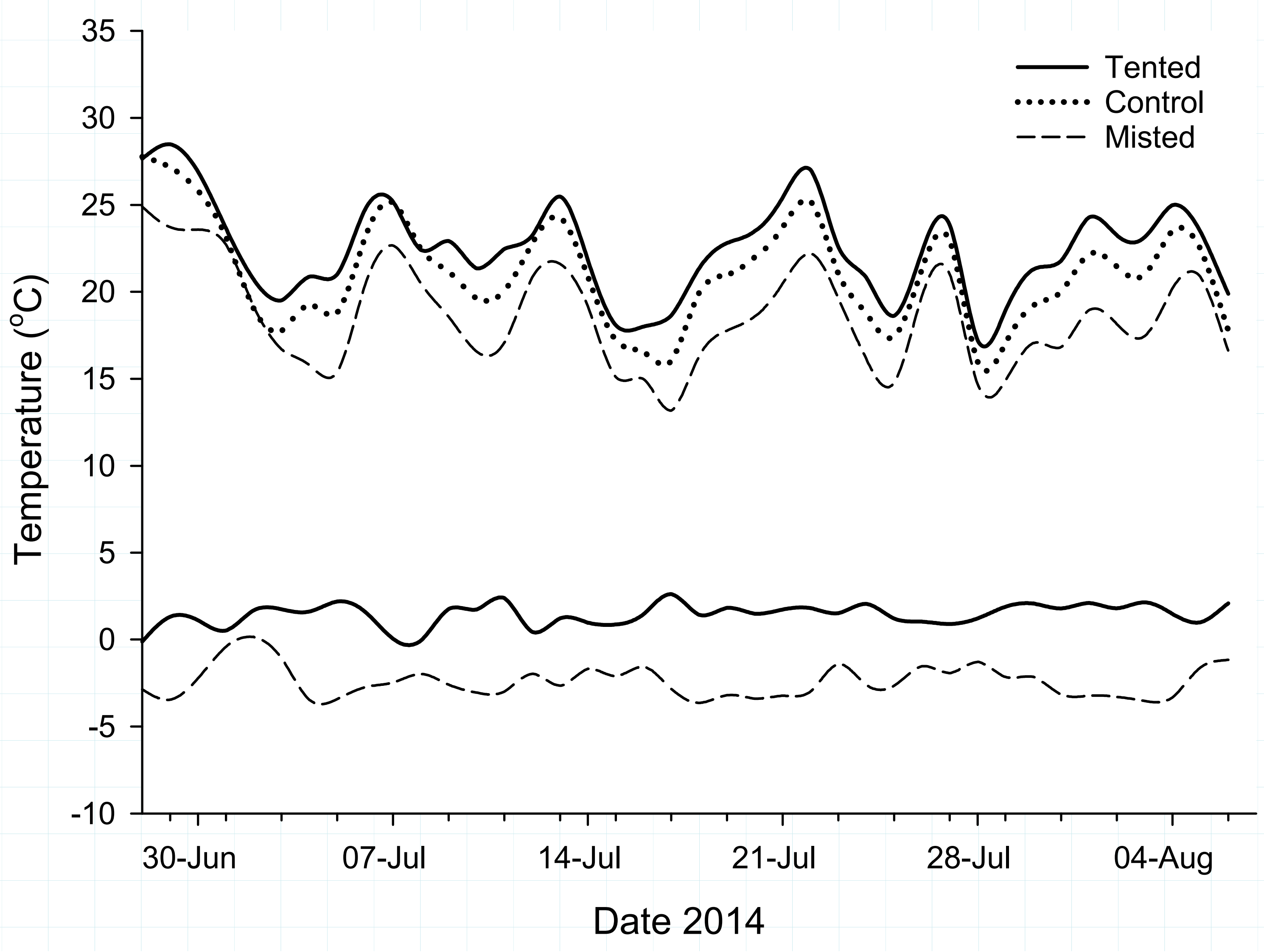


Figure 5.4. Daily maximum and minimum temperatures of shoots of Fraser fir trees that were tented, misted, or control. Minimum temperatures overlap for all treatments.

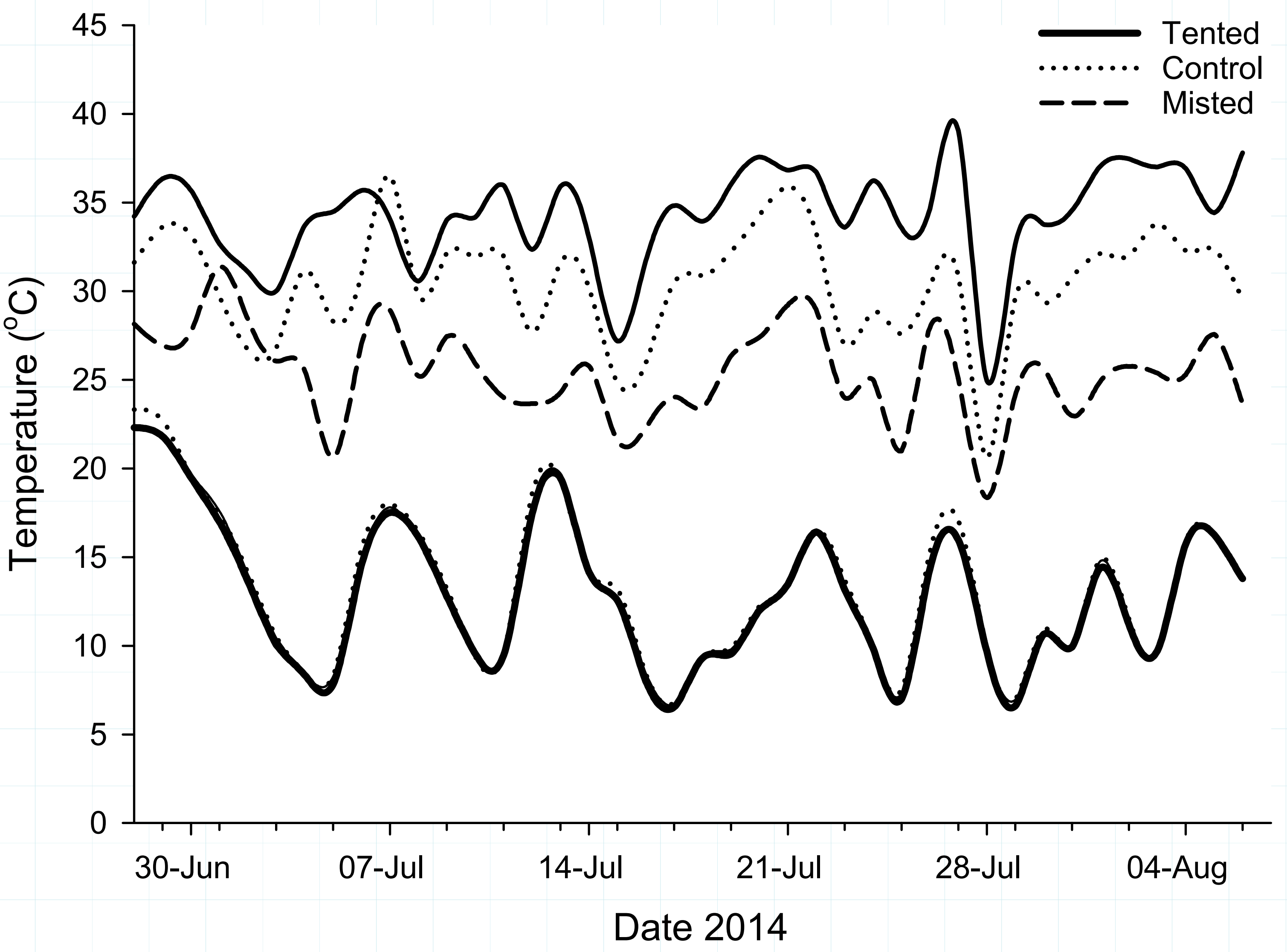


Figure 5.5. Temperatures of shoots of Fraser fir trees that were tented, misted, or control on (a) warm, sunny (July 5); and (b) cool, cloudy (July 28) days in 2014.

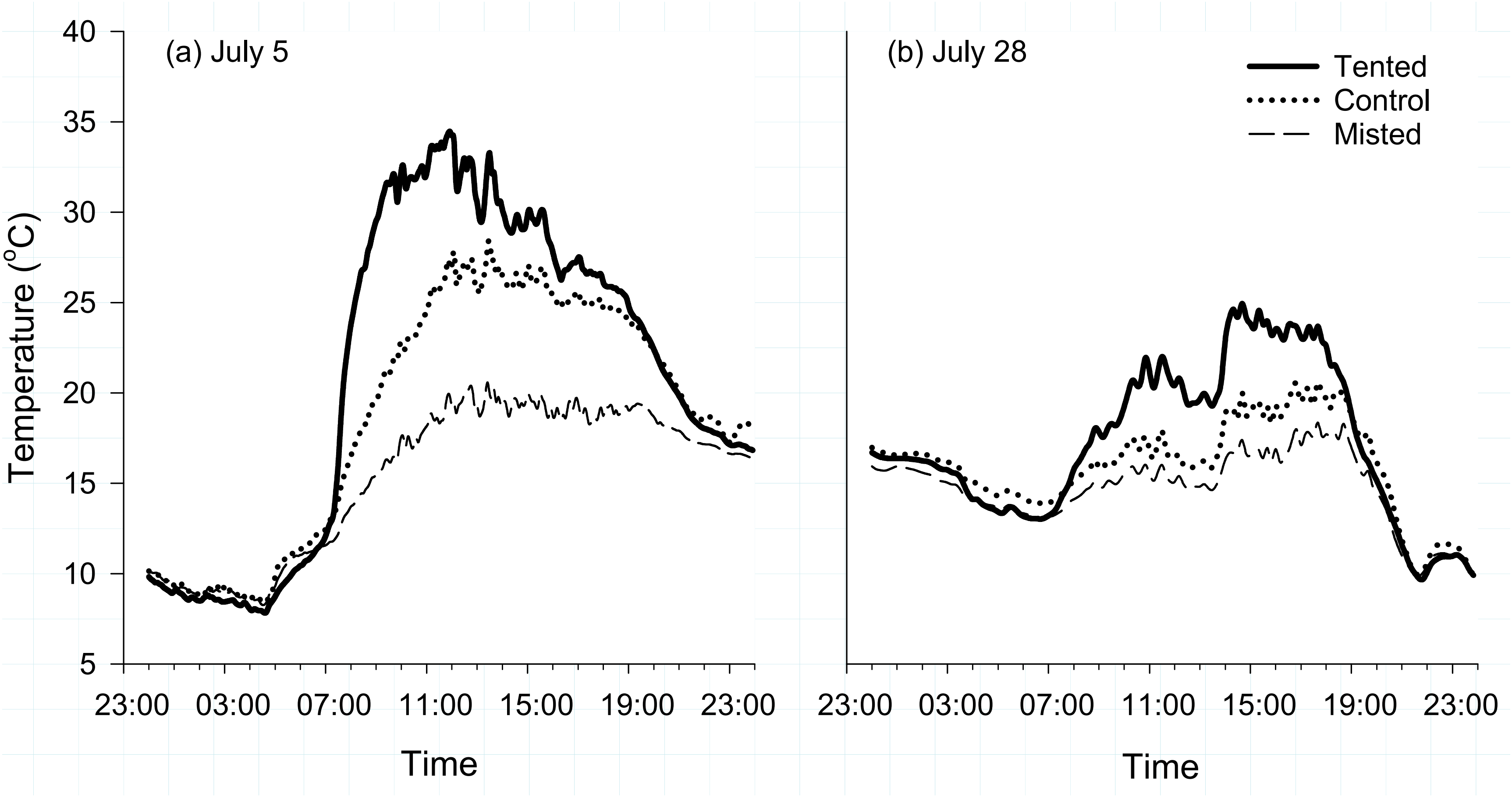


Figure 5.6. Soil moisture under tented, misted, or control Fraser fir trees, 2014.

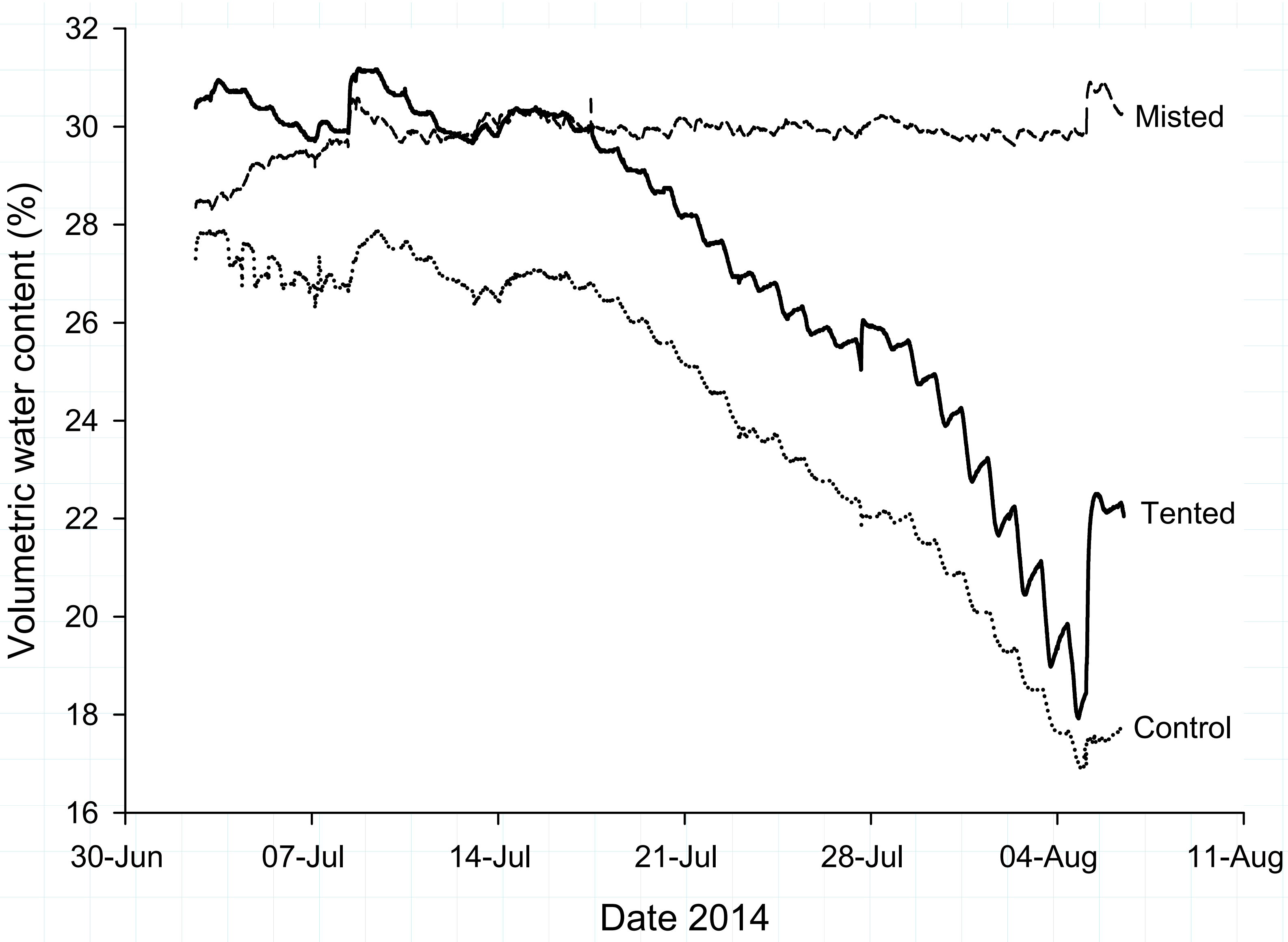


Figure 5.7. Fraser fir tree damage due to intolerance to salts present in well water used for the misting treatment.

CHAPTER SIX

GIBBERELLIC ACID INHIBITORS CONTROL HEIGHT GROWTH AND CONE PRODUCTION IN *ABIES FRASERI*

ABSTRACT

GIBBERELLIC ACID INHIBITORS CONTROL HEIGHT GROWTH AND CONE PRODUCTION IN *ABIES FRASERI*

Precocious cone production in Fraser fir (*Abies fraseri*) results in substantial economic losses for Christmas tree growers in the United States. Cone removal often represents the second highest labor expense, after shearing. Gibberellins (GAs) regulate both reproductive development and shoot elongation, and are used to enhance cone production in conifer seed orchards. We identified plant growth regulators (PGRs) that inhibit GA biosynthesis—paclobutrazol, applied by foliar spray (PBZ-foliar) or soil injection (PBZ-soil), and chlormequat. We evaluated the effects of PGR treatments on cone production and vegetative growth in three tree size classes (small, medium, large) at four locations over a three-year period. PGRs were applied in spring 2013, but did not affect growth until 2014, nor did they affect cone production until 2015. In 2014, PBZ reduced leader length by 21 – 29% across size classes and sites, while also increasing bud density by 11 – 20%. In 2015, PBZ-soil reduced average cone density (cones/tree) in small, medium, and large trees by 33, 54, and 40%, respectively. PBZ-soil also reduced leader length by 20 – 29% and increased bud density by 16 – 25%, making it the most effective PGR at controlling height growth and cone production in Fraser fir.

6.1. INTRODUCTION

In the United States, Fraser fir (*Abies fraseri* [Pursh] Poir.) is considered a premium Christmas tree because of its beautiful form. Natural stands of Fraser fir trees are found only at high elevation sites with abundant rainfall in the southern Appalachian Mountains. Cone production in Fraser fir generally begins when trees are 15 – 20 years old (Johnson 1980). When planted outside of their native range in Christmas tree plantations in the Midwestern United States, Fraser fir trees may produce cones when they are just a few years old (Cregg et al. 2003).

Heavy cone production reduces tree quality and value by decreasing the density of branches in the upper third of the crown. Expanding cones are also strong sinks for photosynthates (Powell 1977; Sala et al. 2012), and may compete with vegetative growth. Therefore, growers typically remove cones by hand early in the growing season. For many growers, cone removal is the second highest labor expense, after shearing.

Most knowledge about manipulation of cone production comes from research designed to increase cone production in seed orchards. Conifer seed production is highly variable from year to year, which is a problem for seed orchard managers who must provide a consistent source of seed to forest nurseries (Owens 1995). Efforts to increase cone production in seed orchards have often relied on cultural treatments, such as girdling, root pruning, fertilization, and induced drought (Puritch 1972). The results of these treatments are highly variable and occasionally detrimental to the health of trees. Subsequent work with plant growth regulators (PGRs), particularly exogenous application of gibberellins (GAs), resulted in greatly enhanced cone production in many species, especially when combined with cultural treatments (Puritch 1979). For example, trunk-injection of $GA_{4/7}$ combined with fertilizer, girdling, and tenting resulted in a 30-fold increase in cone production in Pacific silver fir (*Abies amabilis* [Douglas ex Loudon] Douglas ex Forbes) (Owens et al. 2001). Likewise, endogenous GAs accumulate at the meristem from neighboring tissues immediately prior to strobilus initiation, and regulate organogenesis of reproductive structures (Pharis & Kuo 1977).

Because GAs promote reproductive development in conifers, inhibition of GA biosynthesis may limit cone production. GAs also regulate shoot elongation (Powell 1977). Therefore, GA inhibitors may reduce the need for both cone removal and shearing, which represent the two highest labor expenses for many Christmas tree growers. PGRs—especially

GA inhibitors—are widely used in greenhouse production for height control in a variety of ornamental plants (Larcher et al. 2011). By disrupting GA biosynthesis, GA inhibitors reduce GA-mediated shoot expansion and alter the allocation of photosynthates within a plant. The resultant plants may be more compact, with a decreased shoot to root ratio and increased production of fine root hairs, potentially improving drought tolerance and transplant success (Fernández et al. 2006). The color of flowers and foliage may also be enhanced (Bañón et al. 2002; Lenzi et al. 2015). In the U.S., Christmas trees are often fertilized for improved color, and then heavily sheared by hand to encourage the dense, conical growth form desired by many consumers. GA inhibitors may reduce the need for fertilizer by enhancing color and the need for shearing by reducing leader and lateral branch elongation.

In addition to endogenous control via GA signaling, cone formation may also be regulated by environmental factors such as moisture stress and soil conditions. Drought stress, whether natural or induced, increases cone production in many conifer species (Owens & Blake 1985), such as Norway spruce (*Picea abies* L. Karst.) (Solberg 2004), often as an allocation tradeoff with vegetative growth (Muller-Starck & Seifert 2008). Mulch is commonly used to reduce evaporative loss of water from the soil and may decrease drought stress (van Donk et al. 2011). Some growers routinely apply gypsum to reduce cone production in their fields. The mechanism by which gypsum may decrease cone production is unclear, particularly given that higher nutrition generally increases cone production (Owens 1995). Gypsum may promote changes in calcium signaling, which is important in many cellular functions (Clapham 2007), including plant responses to abiotic stresses (Knight 1999).

In this study, we investigated the effectiveness of cultural approaches for controlling cone production in Fraser fir. Our specific objectives were to evaluate the effects of PGRs on seed cone production and vegetative growth in Fraser fir trees. Secondary objectives were to evaluate the effects of moisture stress (through use of a mulch treatment) and gypsum on cone production and growth.

6.2. MATERIALS AND METHODS

6.2.1. Description of field trials

In May 2013, we established studies at four commercial Christmas tree farms located near Horton (south-central Michigan), Manton (north-central Michigan), Sidney (central Michigan), and Mason (central Michigan) (Table 6.1). As part of ongoing farm operations, trees were fertilized each year and sheared as needed to maintain the desired growth characteristics and shape. We selected a block of trees of similar size at each farm, and excluded trees with no cones from the study. Reproductive trees (those with seed cones in 2013) were divided into three size classes (small, medium, large), the distribution of which varied by location (Table 6.2).

6.2.2. Treatments

At each location, forty small trees were randomly assigned to treatment with PBZ-soil (Cambistat®, paclobutrazol, 200 ml; Rainbow Treecare Scientific Advancements, Minnetonka, MN) or untreated control. Twenty large trees were randomly assigned to treatment with PBZ-soil (600 ml) or untreated control. Twenty medium trees were randomly assigned to one of several treatments or control: 1) PBZ-soil (400 ml); 2) PBZ-soil (600 ml); 3) PBZ-foliar (Trimtect®, paclobutrazol, 50 ml/L; Rainbow Treecare Scientific Advancements, Minnetonka, MN); 4) Chlormequat (Cycocel®, 2000 ppm; OHP, Inc., Mainland, PA); 5) Gypsum ($CaSO_4$, 300 g); 6) $GA_{4/7}$ (ProVide®, 500 ppm, positive control; Valent BioSciences Corporation, Libertyville, IL); 7) mulch (Horton only); or 8) untreated control. $GA_{4/7}$ was included as a positive control because of its promotive effect on cone formation in *Abies* (Owens et al. 2001).

6.2.3. Treatment application

Between May 29 and June 5, 2013, PBZ-soil was injected into the soil at a depth of 8 cm in three equal parts around each treated tree using a pressurized backpack system. Gypsum granules were spread evenly by hand in a ring extending 30 cm outward from the dripline of each tree. Wood chip mulch was applied 5 – 10 cm deep, in a ring extending 45 – 60 cm outward from the drip line of the tree. PBZ-foliar, chlormequat and $GA_{4/7}$ were applied as foliar sprays one time in June 2013 at the end of rapid lateral growth, just prior to reproductive bud initiation (Owens & Blake 1985) (Table 6.2). A backpack sprayer was used to treat the upper half of the crown until all needle and stem surfaces were wet. A non-ionic surfactant (CapSil® Aquatrols, Paulsboro, NJ) was added to all spray treatments (0.5 ml/l solution) to improve coverage and performance. Treatments were not reapplied in subsequent years.

6.2.4. Data collection

We measured tree height and counted seed cones on all trees in the study each May from 2013 – 2015. At the end of each growing season, we measured terminal leader length for all trees and shoot length, needle length, and bud density (buds/cm) for one randomly selected, current-season lateral shoot in the upper few whorls on the south side of each tree.

6.2.5. Tree sampling and stable isotope analysis

In October 2015, we collected one two-year-old lateral branch from upper whorls on the south side of each tree. We divided the shoots by treatment and year into five replicate samples, which were dried at 70°C for three days. Needles were separated, ground to 40-mesh (0.420

mm), and packed in tin capsules. $\delta^{13}C$, the isotopic composition (ratio of ^{13}C to ^{12}C relative to a standard) in needle samples was determined at the Center for Stable Isotope Biogeochemistry at the University of California-Berkeley using an IsoPrime 100 mass spectrometer (Isoprime Ltd., Stockport, UK). From $\delta^{13}C$, we calculated carbon isotope discrimination ($\Delta^{13}C$), using the equation (Farquhar 1989; Cregg & Zhang 2000)

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p}$$

where δ_a is the isotopic composition of air, assumed to be -8‰, and δ_p is the isotopic composition of the plant material, $\delta^{13}C$. $\Delta^{13}C$ provides an integrated measure of moisture stress during active growth. To reduce costs for $\delta^{13}C$ analysis, we only included needle samples from 2014, the year in which more treatments affected growth than in other years. We also limited samples to include only the control; mulch, because of its ability to mitigate drought and heat stress; and PBZ treatments, because of the anticipated impact of PBZ on growth and cone production.

6.2.6. Statistical analysis

6.2.6.1. Effects on cone production

Effects of treatments on cone production were tested by analysis of covariance (ANCOVA) based on a generalized randomized complete block design (GRBD) in PROC MIXED (SAS v9.4; SAS Institute Inc., Cary, NC, USA). Box-Cox transformation ($\lambda = 0.25$) of the response variable (cone density) normalized the distribution of the residuals and equalized variances. Because cone production is positively correlated with tree size, initial tree height at the start of the study was used as a covariate in fitting the model. Data were analyzed by tree size class because different treatments were assigned to each size class. The model was initially fitted using repeated measures (Kenward-Roger adjustment; Toeplitz variance-covariance structure). Because of interactions between year and treatment, height, and site, data were analyzed by year. Site was treated as a random effect to allow prediction of the effects of treatments on cone production across the entire population of Fraser fir trees grown in Christmas tree plantations in the Upper Midwest. Means separation was accomplished using Tukey's honestly significant differences (HSD) at $\alpha = 0.05$.

6.2.6.2. Effects on growth and carbon isotope discrimination

The effects of treatments on growth and carbon isotope discrimination were tested by analysis of variance (ANOVA) based on a GRBD. The model was fitted by size class and year because of 3-way interactions between year, site, and treatment. Site was treated as a random effect to allow generalization of results (particularly those relevant to shearing) across the population of Fraser fir trees grown in Christmas tree plantations in the Upper Midwest. Means separation was accomplished using Tukey's HSD ($\alpha = 0.05$).

6.3. RESULTS

6.3.1. Cone production

Cone production varied widely between years and among farms (Table S6.1). In 2014, trees produced an overall average of 22.5 cones per tree at Horton and 17.2 cones per tree at Sidney. In 2015, trees at these locations produced two to three times as many cones, 68.4 and 35.7 cones per tree, respectively. The trend was the opposite at Mason and Manton, where cone production decreased from one year to the next. In 2014, trees produced 11.2 cones per tree at Mason and 40.4 cones per tree at Manton. In 2015, trees at these locations produced 7.8 and 21.3 cones per tree, respectively.

Treatments did not affect cone production relative to the control in 2014. Compared to the positive control ($GA_{4/7}$), chlormequat reduced the average number of cones per tree by 45% across sites. Both rates of PBZ-soil reduced cone density in medium trees by approximately 65% at Mason, compared to $GA_{4/7}$. In 2015, PBZ-soil reduced the average cone density in small trees by 33%, in medium trees (400 ml rate) by 54%, and in large trees by 40%, across all sites, compared to control (Fig. 6.1).

6.3.2. Growth

For untreated control trees, current-season terminal leader length and needle length increased each year at every site. Leaders were 24% longer on large trees and 10% longer on small and medium trees in 2015 than in 2013. Bud density was highest in 2014. The effects of treatments on growth varied by year but were consistent across locations, except that treatments had no effect on growth at Manton in 2015. Because the effects of treatments were otherwise consistent (i.e., the site x treatment interactions were ordinal), main effects were evaluated across sites.

In 2013, PBZ-soil (400 ml rate) reduced leader growth of medium-sized trees by 16%, but means did not differ from the control for any other treatment (Table 6.3). In 2014, all of the GA inhibitors reduced terminal leader length. Chlormequat reduced leader length by 8%. PBZ-soil and PBZ-foliar reduced leader length by 21 – 29% across size classes, while also increasing bud density by 11 – 20%, and reducing needle length of small and medium trees by 7 – 12%. In 2015, PBZ-soil reduced leader length by 20 – 29%, increased bud density by 16 – 25%, and reduced needle length of small and medium trees by 5 – 8%.

6.3.3. Carbon isotope discrimination and nutrient analysis

The effects of treatments on $\Delta^{13}C$ were generally consistent across size classes and locations. PBZ-soil and PBZ-foliar reduced $\Delta^{13}C$ in all size classes across farms in 2014, compared with control (Table 6.3). However, PBZ-soil did not affect $\Delta^{13}C$ in large trees at Sidney or in small trees at Manton and Horton. Mulch did not affect $\Delta^{13}C$ in any size class.

6.4. DISCUSSION

Cone formation in conifers is a complex phenomenon controlled by a range of factors, both environmental and endogenous (Day & Greenwood 2011). In our study, cone density was regulated by both exogenous and endogenous factors. Although the effects of PBZ were not immediately apparent, its suppression of both cone formation and shoot elongation may be of benefit to Christmas tree growers. By 2015, mean cone density was 39% lower in trees treated with PBZ (either -foliar or -soil) than in control trees, averaged across all locations and size classes. Even in 2014, PBZ-soil appeared to be physiologically active in reducing cone development in medium trees at one location (Mason), evidenced by the statistical separation in means of trees treated with $GA_{4/7}$ (positive control) and those treated with PBZ-soil (Table S6.1).

In general, spray treatments had little effect on cone production in 2014, likely because of genetic variability, product rate, and timing of application. We observed high inter-tree variation in both cone production and growth, and attempted to control for this genetic variability by using size classes in the study design and initial height as a covariate in the analysis. However, the power to statistically separate the effects of treatments was reduced because of this inter-tree variation.

The efficacy of PGRs in controlling cone production in trees of any size could likely be improved by increasing the application rate. Adjusting the rate for each tree is not practical in a production setting, so we followed the manufacturer's recommendations based on the range of tree sizes in each size class. However, effective rates have not been determined for Christmas trees and other nursery crops, which are intermediate in size between the floriculture and arboriculture plants for which rates are known. There was no incidence of phytotoxicity observed during this study, but it may be a problem at higher product rates.

The window of bud initiation and differentiation in conifers varies from year to year, because reproductive phenology is based on thermal time, rather than calendar date. To overcome this difficulty, GA sprays are generally applied several times at 7 – 10 day intervals to increase cone production in seed orchards (Owens & Blake 1985). For example, in *A. amabilis*, it is recommended to begin spray treatments at vegetative bud break (Owens et al. 2001). However, mean cone density was higher in trees treated with GA at all locations in our study—57% higher than control, on average. Although not detectible in our statistical tests, this increase probably resulted from the activity of GA in promoting reproductive development, and suggests that timing of PGR application coincided with cone bud formation. A second application, 7 – 10 days later, may improve the effectiveness of treatments. Interestingly, trees treated with GA produced more cones than control trees in 2014, but fewer in 2015. Although cones were removed from the trees in late spring, stimulation of cone production by GA appears to have suppressed subsequent cone production, perhaps by a mechanism that regulates mast-seeding in *Abies* (see Koenig & Knops 2000; Sala et al. 2012). This may have implications for seed orchard managers, who require consistent cone production each year. However, annual application of GA may overcome this problem.

Not surprisingly, treatments had minimal effect on growth parameters in the year of application. Sprays were applied just prior to reproductive bud initiation, which occurs after most of the vegetative growth is complete. The soil-injected PBZ-soil, although applied early in the season, requires some time to be taken up by the roots and distributed throughout the tree. All of the GA inhibitors demonstrated a carryover effect by reducing leader growth in all size classes the year following application (Table 6.3). PBZ-soil demonstrated the highest and most persistent growth effect, reducing leader growth for all size classes in 2014 and 2015. All of the trees were on operational farms and were sheared each year as needed. Terminal leaders are generally sheared to around 30 – 38 cm, depending on tree size and shape. Because PBZ reduced leader growth to under 38 cm for most trees in 2014 and 2015, many trees required only minimal shearing, and often the leader could be left intact, preserving the terminal buds. Slightly higher rates of PBZ could be used to further reduce growth, bringing average leader length closer to 30 cm. By reducing shoot length, PBZ also increased bud density, which could result in fuller trees, which are desirable to consumers in the United States.

$\Delta^{13}C$ varies primarily because of discrimination against $^{13}CO_2$ during photosynthesis, first during diffusion across the stomatal pore, and later by ribulose-1,5-bisphosphate carboxylase (RuBisCo) during carbon fixation (Farquhar 1989). Induction of stomatal closure is one of the physiological mechanisms by which PBZ increases drought tolerance in conifers (Marshall et al. 1991). PBZ may also increase chlorophyll content of conifer needles (Mahoney et al. 1998). Therefore, the decrease in $\Delta^{13}C$ observed in trees treated with PBZ-soil or PBZ-foliar may result from a reduction in stomatal conductance or from an increase in carbon assimilation. Overall, total net carbon gain is likely to be reduced in trees treated with PBZ, since the photosynthetic efficiency (increased chlorophyll content) is unlikely to offset the effects of reduced stomatal conductance and decreased effective leaf area (reduced shoot and needle length). Therefore, PBZ may reduce cone production by reducing available GA at the lateral bud through direct interference with GA biosynthesis, or by decreasing reproductive bud survival by reducing the carbon required for continued bud development (Ebell 1971).

Mulch did not affect $\Delta^{13}C$, suggesting that soil moisture was adequate for both mulched and control trees in 2014. In the absence of drought stress, it is not surprising that mulch did not reduce cone density the following spring. However, conifers native to regions with high rainfall generally exhibit inefficient stomatal closure even under drought conditions (Brodribb et al. 2014). Therefore, it may be difficult to quantify the level of relative drought stress using $\Delta^{13}C$, except under extreme and prolonged drought.

PBZ-soil was the most effective and persistent treatment over a three-year time period for control of both cone formation and tree height in Fraser fir. Christmas tree producers may obtain desirable results, including increased bud density and reduced costs for shearing and cone-removal, by applying PBZ by soil injection or soil drench. Although PBZ-soil is expected to control growth for three to five years, persistence in the soil will vary based on rate and site-specific factors, including soil type and management practices. For example, the effects of PBZ-soil on growth were no longer apparent in the third year after application at Manton, in contrast to all other locations. Conifers require relatively high product rates to see an effect with PBZ (Wheeler 1987). Producers should consult with the product manufacturer to determine product half-life in their fields, to reduce undesired carryover effects in the next rotation. Producers may also want to experiment with PBZ-foliar, applying it by spray each year in early summer, when lateral shoot elongation is beginning to slow.

Apart from PBZ, we cannot recommend any of the tested treatments for control of cone production and height growth in Fraser fir, based on the results of this study. Although chlormequat reduced average cone density compared to $GA_{4/7}$ in 2014, results were inconsistent across sites and it lacked the growth control and persistence apparent with PBZ. Neither gypsum nor mulch affected average cone density or growth.

In conclusion, PBZ reduced cone production by 39% in Fraser fir trees grown in Christmas tree plantations across all sites and size classes, while simultaneously reducing the need for shearing in plantation systems. If product were applied at slightly higher rates, these benefits could likely be improved, although the risk of phytotoxicity may increase. Mulch did not affect cone production or growth in any year, but trees were not subjected to prolonged drought conditions because of adequate precipitation. Likewise, gypsum did not affect growth or cone production, and cannot be generally recommended for control of cone production. Finally, GA may be useful in promotion of cone development in *Abies* seed orchards, but cone production may be lower the year following a heavy cone crop.

APPENDIX

Table 6.1. Site characteristics of study locations in Michigan, 2013.

	Coordinates (decimal degrees)		Soil [a]			Mean daily temperature[b] (°C)		Total precipitation [b] (cm)	
City	Latitude	Longitude	Type	Slope (%)	pH	Annual	June	Annual	June
Horton	42.0760	-84.4830	Hillsdale-Riddles sandy loams	6 – 12	6.2	10.5	19.8	80.0	8.3
Manton	44.3986	-85.2875	Montcalm-Graycalm complex	0 – 6	5.8	9.0	18.4	87.2	9.0
Mason	42.6661	-84.4482	Capac loam	0 – 4	6.6	10.4	19.8	81.7	8.9
Sidney	43.3027	-85.0248	Tekenink-Elmdale loamy sands	2 – 6	5.8	9.8	19.3	83.9	8.7

a. Web soil survey (USDA NRCS).

b. 1981 – 2010 U.S. Climate Normals (NOAA 2010).

Table 6.2. Distribution of trees (range [mean]) in each size class and dates of PGR application at study sites in Michigan, 2013.

	Size class (m) [a]			Date 2013		
Site	Small	Medium	Large	PBZ soil injection	Foliar sprays	Gypsum
Horton	1.0 – 1.6 (1.4)	1.6 – 2.0 (1.8)	2.0 – 2.5 (2.1)	5 Jun	19 Jun	5, 11 Jun
Manton	1.0 – 1.3 (1.2)	1.3 – 1.5 (1.4)	1.5 – 1.8 (1.6)	4 Jun	20 Jun	4 Jun
Mason	1.0 – 1.3 (1.2)	1.3 – 1.6 (1.4)	1.6 – 2.0 (1.7)	29 May	18 Jun	7 Jun
Sidney	1.0 – 1.3 (1.2)	1.3 – 1.7 (1.5)	1.7 – 2.0 (1.8)	29 May	20 Jun	13 Jun

a. Tree sizes varied by location. Trees with cones were assigned to size classes to yield 80 small, 120 medium, and 40 large trees per site.

Table 6.3. Mean shoot growth, bud density, and carbon isotope discrimination of Fraser fir trees by size class and treatment across four locations in Michigan, 2013 – 2015.

		Leader Length (cm)			Needle Length (mm)			Bud Density (buds/cm)			$\Delta^{13}C$ (‰)
Size Class	Treatment	2013	2014	2015	2013	2014	2015	2013	2014	2015	2014
Small [a]	PBZ-soil (200ml)	41.3	33.0b	36.7b	14.2	14.2b	15.5b	0.52	0.65a	0.60a	21.11b
	Control	41.5	44.3a	46.1a	14.2	15.3a	16.5a	0.52	0.58b	0.51b	21.27a
Medium [b]	PBZ-soil (400 ml)	38.8b	34.5d	36.5b	14.0	14.7cd	15.6bc	0.50ab	0.64a	0.59ab	21.13b
	PBZ-soil (600 ml)	40.8ab	33.6d	35.9b	14.3	13.9d	15.1cd	0.54a	0.65a	0.64a	20.95c
	PBZ-foliar	43.0ab	33.7d	48.4a	14.0	14.3cd	16.4ab	0.54a	0.61ab	0.55bc	21.10b
	Chlormequat	43.0ab	43.4c	52.9a	14.5	17.4a	14.6d	0.47b	0.52d	0.51c	†
	Gypsum	42.1ab	44.0bc	48.6a	14.1	15.6b	17.0a	0.50ab	0.56cd	0.52c	†
	Mulch	†	41.6bc	†	14.7	15.6bc	17.7a	0.45ab	0.55bd	†	21.29ab
	$GA_{4/7}$	43.5ab	47.6a	48.5a	14.0	15.9b	16.8a	0.52ab	0.57bc	0.51c	†
	Control	46.2a	47.1ab	50.9a	13.9	15.8b	16.5ab	0.52ab	0.55cd	0.51c	21.31a
Large [b]	PBZ-soil (600 ml)	39.4	35.3b	37.1b	13.8	15.1	16.6	0.57	0.66a	0.62a	20.93b
	Control	40.4	44.5a	50.2a	13.7	15.1	16.7	0.53	0.55b	0.51b	21.18a

Note: Different letters within the same column and size class indicate significant differences at $\alpha = 0.05$ (Tukey's HSD).

a. 60 samples/treatment for needle length, 160 for leader length and bud density.

b. 60 samples/treatment for needle length, 80 for leader length and bud density.
† Not sampled.

Supplemental Table S6.1. Mean cone density (cones/tree) on Fraser fir trees by size class and treatment at four locations in Michigan.

		Manton		Horton		Sidney		Mason	
Size Class	Treatment	2014	2015	2014	2015	2014	2015	2014	2015
Small[a]	PBZ-soil (200ml)	16.6	12.0	11.8	39.9**b**	6.1	10.1	2.0	4.1
	Control	16.2	13.2	8.4	65.2**a**	3.0	13.2	5.6	6.4
Medium[b]	PBZ-soil (400 ml)	36.9	12.2	26.7	46.9	13.6	22.3	7.8**b**	7.2
	PBZ-soil (600 ml)	45.3	18.2	15.7	76.2	15.1	38.9	7.5**b**	6.0
	PBZ-foliar	51.9	18.8	28.9	66.0	15.2	21.5	16.8	11.2
	Chlormequat	42.5	32.5	31.0	137.7	5.1	45.2	12.5	4.8
	Gypsum	41.2	36.4	31.6	65.5	19.8	32.6	7.7	13.6
	Mulch	†	†	18.6	69.5	†	†	†	†
	$GA_{4/7}$	53.7	18.0	27.4	42.4	43.7	31.8	21.7**a**	7.0
	Control	50.2	31.0	17.4	84.8	14.8	57.9	10.7	11.9
Large[b]	PBZ-soil (600 ml)	63.4	11.6**b**	30.8	81.8	55.2	71.3	20.7	6.0
	Control	75.2	47.9**a**	47.7	138.1	38.5	130.2	24.2	12.0

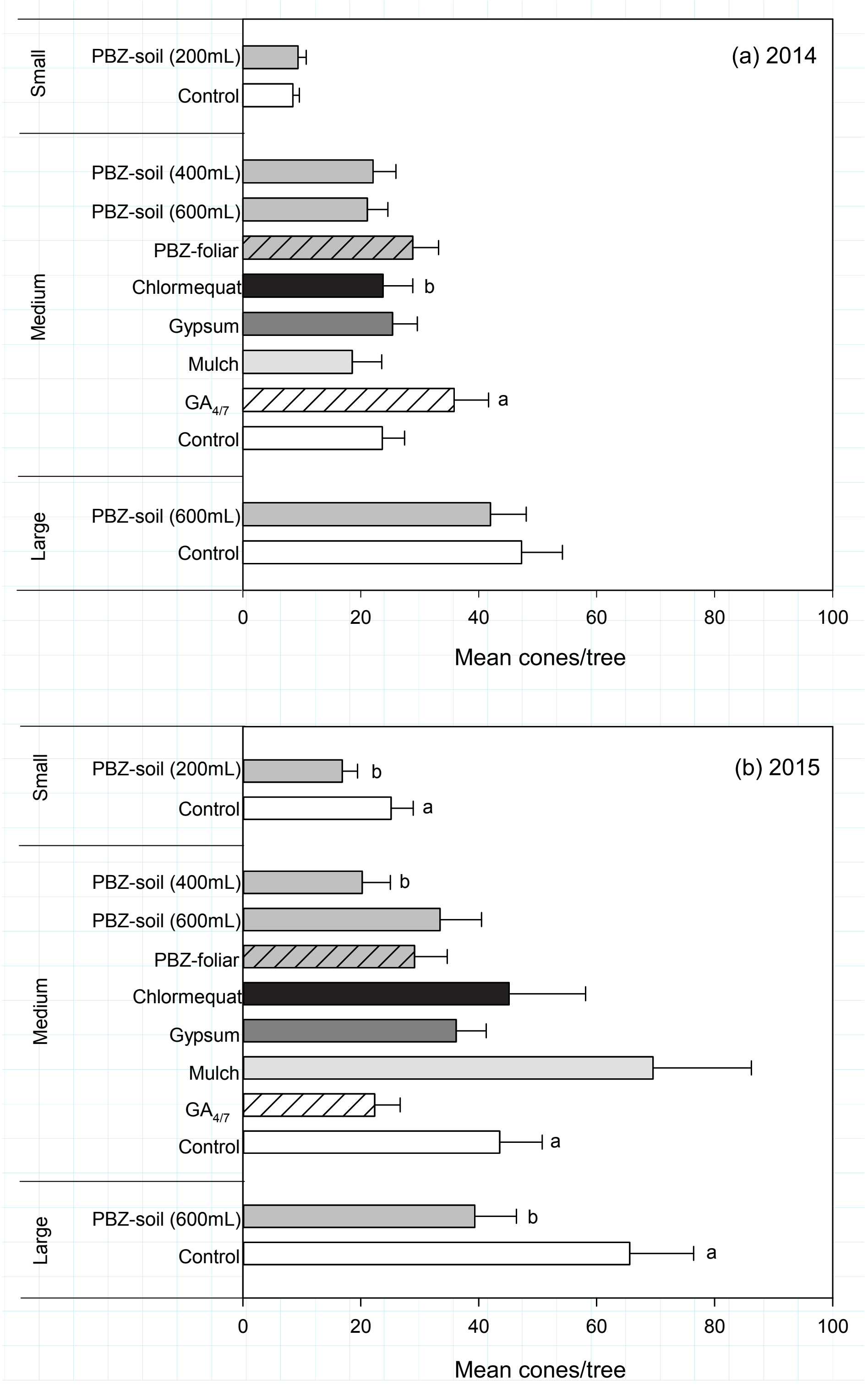
Note: Different letters within the same column and size class indicate significant differences at $\alpha = 0.05$ (Tukey's HSD).

a. Samples per treatment at each site = 40.

b. Samples per treatment at each site = 20.

† Not sampled.

Figure 6.1. Mean cone density (cones/tree) on small, medium, and large Fraser fir trees in (a) 2014 and (b) 2015 by treatment across four locations in Michigan. Different letters within the same size class indicate significant differences at α = 0.05 (Tukey's HSD). N = 160 samples per treatment for small trees; 80 samples per treatment for medium and large trees.



CHAPTER SEVEN

OVERALL CONCLUSION

This research extends what is known about the regulation and timing of reproductive development in *Abies*, and is important for Christmas tree producers and seed orchard managers. It may also be helpful to forest managers. Fraser fir trees grow in sensitive, high elevation ecosystems and, like many *Abies* species, are threatened in their native range (IUCN 2012). Changes in environmental factors and ecosystem management have been shown to impact the health and survival of Fraser fir trees (Swanson 2012). Decoding the complex interaction between the environment and conifer reproduction will be crucial for forest management decision-making to ensure the long-term health and survival of Fraser fir and other *Abies* species.

Manipulation of cone production in *Abies* remains challenging. However, our phenology model of shoot growth represents a significant advancement in understanding the timing of reproductive development in Fraser fir, and the results from our irrigation, mulch, misting, and PGR studies provide some clarity on environmental and chemical control of cone formation. Previously, the timing of reproductive bud initiation and differentiation was only vaguely known, and growers adjusted management practices based on anecdotal evidence in the attempt to reduce cone production in their fields. Our phenology model is able to predict the timing of shoot growth and reproductive bud initiation and differentiation, and is robust to extremes of temperature and precipitation. It is available to Christmas tree producers and seed orchard managers online at http://enviroweather.msu.edu/. Work is underway to incorporate additional information into the online management tool, including guidance on timing of application of herbicides and sprays for control of balsam twig aphid (*Mindarus abietinus* Koch [Homoptera: Aphididae]). We did not perform our own anatomical work to determine the timing of reproductive bud initiation and differentiation, which would have strengthened our confidence in the model. However, our model is based on anatomical work done in closely related *Abies* species that should generalize to Fraser fir (see Owens & Blake 1985).

Our results demonstrate that it is difficult, yet indeed possible, to influence cone production in Fraser fir by cultural and chemical means. This research improves our basic understanding of reproductive biology in *Abies*, and clarifies the role of environmental factors in reproductive development of Fraser fir trees grown in plantations. Results from our irrigation studies indicate that cone production is regulated by factors other than available soil moisture, and unwanted cones cannot be limited by irrigation as was previously recommended—at least under the conditions at our research sites during these studies. Our mulch studies suggest that soil temperatures regulate cone production, but the effects of mulching on cone formation are likely to be variable, with a reduction in cones observed only subsequent to very hot weather during strobilus bud initiation and differentiation. Altering shoot temperature by mist cooling or tenting of trees did not affect cone production, suggesting that temperatures at the lateral shoot do not regulate cone formation. However, it is unclear whether cooler temperatures might have suppressed cone production, or whether the accumulation of warm temperatures over several years has advanced tree maturity, resulting in higher cone production at a younger age and size. The results of our PGR studies indicate that GA inhibitors, particularly paclobutrazol applied to the soil, may effectively control cone formation in Fraser fir. These results are similar to those noted in research related to cone induction or enhancement in conifer seed orchards: GA is the most effective treatment for cone induction, and the effects of GA are often enhanced by adjunct cultural practices that may produce variable results or show no effect apart from GA (Owens & Blake 1985; Bonnet-Masimbert 1987; Pharis et al. 1987).

To better understand the reproductive biology, future studies should examine the effects of (1) further cooling of the roots and shoots, (2) the accumulation of tree stress (heat and moisture stress) over several years beginning with very small trees, and (3) the use of cultural practices in combination with PGRs (e.g., paclobutrazol + mulch; paclobutrazol + misting; GA + tenting). Irradiance is also associated with cone development in conifers (Kosiński & Giertych 1982; Despland & Houle 1997; Koenig & Knops 2000), and its effects on cone formation could also be explored, such as through installation of shade cloth or overstory planting.

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